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OBSERVATIONS ON THE BACTERICIDAL AND BACTERIOSTATIC ACTIONS OF *P*.AMINOBENZENESULPHONAMIDE AND *P*.-HYDROXYLAMINOBENZENESULPHONAMIDE, WITH SPECIAL REFERENCE TO THEIR SUPPRESSION BY *P*.AMINOBENZOIC ACID.

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In previous papers (Burton, McLeod, McLeod and Mayr-Harting, 1940; and Burton, McLeod, Mayr-Harting and Walker, 1942) observations have been published bearing on Mayer's (1937) suggestion that the oxidation of sulphanilamide in the body was necessary to the development of its bacteriostatic and bactericidal effects. In the first paper it was noted that *in vitro* only a limited number of bacteria were markedly sensitive to sulphanilamide and its various substitution products, such as sulphapyridine. These were the Neisseria (notably the gonococcus and meningococcus), the diphtheria bacillus and the pneumococcus, the first two of these being consistently sensitive to sulphonamides, but the last more irregularly so. By contrast all bacteria were sensitive to *p*.hydroxylaminobenzenesulphonamide *in vitro*, and many insensitive to sulphanilamide were found to be markedly sensitive to *p*.nitrobenzenesulphonamide. Further, there was a considerable but incomplete correlation between the oxidizing and reducing activities of bacteria and their sensitiveness to sulphanilamide *in vitro*, e.g. the meningococcus, outstanding amongst bacteria in its capacity to oxidize phenylene diamines and a very strict aerobe, was peculiarly sensitive, whereas the anaerobes and other bacteria endowed with powerful reducing mechanisms were relatively insensitive. There were exceptions, however, since the cholera vibrio, endowed with a mechanism for oxidizing phenylene diamines very similar to that of the meningococcus, was relatively insensitive to sulphanilamide although markedly sensitive to *p*.nitrobenzenesulphonamide. In the second paper strong presumptive evidence that *p*.nitrobenzenesulphonamide was reduced by all bacteria in greater or less degree to *p*.hydroxylaminobenzenesulphonamide was brought forward. No direct evidence for the oxidation of sulphanilamide by bacteria has as yet been obtained however.

Since Mayer's work a new orientation has been given to thought on the mechanism of sulphonamide action by the discovery of Woods (1940) that *p*.aminobenzoic acid markedly antagonizes the action of sulphanilamide. This discovery threw light on the observations of Stamp (1939) and Green (1940) on the interference with sulphonamide action exhibited by extracts of bacterial cultures. Woods' observation has

been confirmed on all sides, and Fildes (1940) has elaborated the theory suggested that *p*.aminobenzoic acid is an essential food factor for bacteria, and that sulphanilamide, resembling it closely in constitution, is effective through blocking some enzyme necessary to the chain of metabolic reactions, involving this essential food factor. If this is in fact the whole explanation of sulphonamide action, a theory of activation of sulphonamide by oxidation becomes superfluous, and Green and Bielschowsky in more recent work (1942) maintain that *p*.hydroxylaminobenzenesulphonamide is unlikely to be responsible for the action of sulphanilamide since its bactericidal effect is not inhibited by *p*.aminobenzoic acid. Further, they have demonstrated the marked bactericidal action of *p*.aminothiophenol and 4 : 4′diamino-diphenyl-disulphide which may be considered rather as reduction products of *p*.aminobenzenesulphonamide.

When working with the meningococcus and pneumococcus we were not able to corroborate fully Green and Bielschowsky's observations made with other bacteria that *p*.aminobenzoic acid does not interfere with the bactericidal activity of *p*.hydroxylaminobenzenesulphonamide. Its interference with the latter compound, although much less than that with *p*.aminobenzenesulphonamide, was still distinct. Since, however, their observations were carried out with the low melting-point form of *p*. hydroxylaminobenzenesulphonamide (M.P. 140° C.), and ours like those of Mayer (1937) with the high melting-point form (161° C.), it was necessary to examine first the discrepancy between the low and high melting-point forms. Burton (1941) had supposed these to be dimorphic forms of the same compound, but Sevag (1943) brought forward evidence to show that in fact the "low melting-point form" was *p*.hydroxylaminobenzenesulphonamide, and that the "high melting-point form" was a molecular complex consisting of two molecules of the hydroxylamine compound with one of sulphanilamide. Burton and Walker (1943) have examined the matter afresh and confirmed Sevag's conclusion with additional evidence. If the *p*.hydroxylaminobenzenesulphonamide portion of the complex is responsible for its superiority as a bactericidal agent to *p*.aminobenzenesulphonamide, it should follow that *p*.hydroxylaminobenzenesulphonamide would be found more actively bactericidal than the molecular complex of the two substances. Further, the partial interference by *p*.aminobenzoic acid with the action of the molecular complex (high M.P. form of *p*.hydroxylaminobenzenesulphonamide) might be entirely due to its contained sulphanilamide, and it might well be that the discrepancy between our results and those of Green and Bielschowsky had been due to the *p*.aminobenzenesulphonamide content of our preparations. On investigation it was found as expected that the bactericidal activity of *p*.hydroxylaminobenzenesulphonamide (M.P. 140° C.) was rather greater than that of the molecular complex (M.P. 161° C.), except in the case of such bacteria as had already been shown to be at least as sensitive to *p*.aminobenzenesulphonamide as to the complex, i.e. the meningococcus, the pneumococcus and the *C. diphtheriae*.

The relative potencies of the high melting-point complex on the one hand and of *p*.hydroxylaminobenzenesulphonamide (M.P. 140° C.) on the other were compared, using five micro-organisms.

Comparison of the Bactericidal Activities of p.Hydroxylaminobenzenesulphonamide and of the "Complex."

The micro-organisms used for this investigation were : The meningococcus (two strains), the pneumococcus (four strains), the streptococcus (four strains), *B. coli* (one strain) and *C. diphtheriae* (one strain of each of the three types). The results obtained were as follows :

TABLE I.

Bacterium.	Strain.	Experiment.	Highest titre preventing growth completely. *p*.hydroxylamino-benzenesulphonamide.	"Complex."
Meningococcus	I	I	40,000	40,000
,,	I	II	>300,000	>300,000
,,	I	III	400,000	400,000
,,	II	I	200,000	80,000
,,	II	II	250,000	250,000
,,	II	III	400,000	400,000
Pneumococcus	I	I	3,000	6,000
,,	II	I	3,000	20,000
,,	III	I	4,000	12,000
,,	IV	I	24,000	24,000
Streptococcus	Richards	I	6,000	1,500
,,	Chestle	I	3,500	3,000
,,	III	I	7,000	6,000
,,	IV	I	8,000	6,000
B. coli	I	I	>2,000	>2,000
,,	I	II	1,500	<1,000
C. diphtheriae	I *gravis*	I	20,000	20,000
,,	II *mitis*	I	20,000	24,000
,,	III *inter.*	I	30,000	35,000

The results obtained are much as expected. In one experiment with *B. coli* an end-point was not reached with either substance; in the other the *p*.hydroxylaminobenzenesulphonamide proved superior. With four different strains of streptococcus the *p*.hydroxylaminobenzenesulphonamide was found to be regularly a little more active than the "complex." With the pneumococcus and *C. diphtheriae*, on the other hand, bacteria specially sensitive to sulphanilamide, the "complex" proved slightly more active than the *p*.hydroxylaminobenzenesulphonamide. In the case of the meningococcus there was one inconclusive experiment, one in which the *p*.hydroxylaminobenzenesulphonamide proved more bactericidal and four in which there was no difference. This would suggest that the meningococcus was about equally susceptible to *p*.hydroxylaminobenzenesulphonamide and *p*.aminobenzenesulphonamide. In our earlier work (Burton *et al.*, 1940), in which the observations on meningococcus had been made on media containing blood, it had appeared that *p*.aminobenzenesulphonamide was more active than the complex against that organism. In view, however, of the readiness with which *p*.hydroxylaminobenzenesulphonamide and blood pigment interact (Burton *et al.*, 1942), it may well be that *p*.hydroxylaminobenzenesulphonamide action is partly suppressed in such media. The point was therefore reinvestigated comparing *p*.hydroxylaminobenzenesulphonamide and *p*.aminobenzenesulphonamide directly in serum agar media for suppression of growth of meningococcus. In one experiment *p*.hydroxylaminobenzenesulphonamide proved slightly superior with one of two strains investigated. No difference was observed in the second and third experiments. It would seem, therefore, that the comparative bactericidal titres of *p*.hydroxylaminobenzenesulphonamide and of the "complex" fully bear out the chemical findings with regard to the composition of the second compound if the respective bactericidal activities of *p*.aminobenzenesulphonamide and *p*.hydroxylaminobenzenesulphonamide are taken into account.

Comparison of the Suppression of the Bactericidal Effects of p.*Aminobenzenesulphonamide and* p.*Hydroxylaminobenzenesulphonamide by* p.*Aminobenzoic Acid.*

Green and Bielschowsky (1942) have stated categorically that their results show that the hydroxylamino-compound (*p*.hydroxylaminobenzenesulphonamide) has not the properties of an active compound since it is not antagonized by *p*.aminobenzoic acid. Earlier results of ours with the supposed high melting-point form of *p*.hydroxylaminobenzenesulphonamide had conflicted with this finding, but for the reasons already set out above all these results have been discarded, and the question has been reinvestigated with pure *p*.hydroxylaminobenzenesulphonamide (M.P. 140° C.). The above authors also claim that statements appearing in the literature on the effective minimal bactericidal concentration of substances tested are valueless when the size of the bacterial inoculum is not accurately standardized. This is only true when the substances are not compared in the same experiment, and in any case we believe that when several dilutions of the inoculum are tested and the actual number of colonies growing in the control are determined, the results are actually more reliable than in experiments with a measured inoculation, since the actual vigour of the bacteria will vary from experiment to experiment however carefully " standardized " the inoculum may be. For example, in one of the tables in the work of Green and Bielschowsky, a differentiation is made between substances which prevent the growth of an inoculum of two bacteria per ml. and others which only retard the growth of such an inoculum. Unless these substances are compared in one and the same experiment this seems to us to be a refinement of differentiation which is scarcely justifiable unless a very large number of experiments have been made with identical result.

Woods (1940) found that the bacteriostatic action of $3{\cdot}03 \times 10^{-4}$ M. sulphanilamide on a strain of group A haemolytic streptococcus growing in a chemically defined medium could be reversed by the addition of a final concentration of from $1{\cdot}2\text{–}5{\cdot}8 \times 10^{-8}$ M. *p*.aminobenzoic acid. This indicates a ratio of 1 molecule of *p*.aminobenzoic acid to 5700–28,900 molecules of sulphanilamide. Also by using *B. coli* in Fildes' lactate medium he obtained a *p*.aminobenzoic acid/sulphanilamide ratio of 1/5200. Many other workers have confirmed and extended these observations. Thus Rubbo and Gillespie (1940) found the antagonistic ratio of *p*.aminobenzoic acid to sulphanilamide to be 1/23,000 for *Cl. acetobutylicum* in a synthetic medium. These authors claimed that *p*.aminobenzoic acid was the sole growth factor required by several strains of *Cl. acetobutylicum*, but Park and Wood (1942) and Lampen and Peterson (1941) showed that this organism needs a mixture of biotin and *p*.aminobenzoic acid for growth in synthetic medium.

Strauss, Lowell and Finland (1941), using type III pneumococcus in a beef infusion broth medium containing 0·05 per cent. dextrose, 1 per cent. Difco peptone and 1 per cent. defibrinated rabbit blood, found that the bacteriostatic action of 10 mg. per cent. sulphapyridine and of 10 mg. per cent. sulphanilamide was removed by ·02 and ·004 mg. per cent. *p*.aminobenzoic acid respectively. The *p*.aminobenzoic acid/drug ratios were found to be 1/250 for sulphathiazole, 1/500 for sulphapyridine and 1/2500 for sulphanilamide.

Wyss (1941) measured the growth rates of *B. coli* in a synthetic medium in the presence of various concentrations of sulphanilamide and *p*.aminobenzoic acid, and, using the half-maximum growth rate as a critical end-point, obtained the value of 1 : 2000 for the *p*.aminobenzoic acid/sulphanilamide antagonistic ratio.

Rantz (1942) found the antagonistic ratio of *p*.aminobenzoic acid/sulphanilamide to be 1 : 2500 for *Staphylococcus aureus* and *B. coli* in simple media, using a photometric method for measuring growth. Using *B. coli* in a synthetic medium, Rose and Fox (1942) determined the following *p*.aminobenzoic acid/drug ratios: For

TABLE II.

Pneumococcus 2. Pneumococcus 18. Pneumococcus 3501. Pneumococcus 1634.

Table II.

	Pneumococcus 2.			Pneumococcus 18.			Pneumococcus 3501.			Pneumococcus 1834.		
	Heavy inoc.	1/50 inoc.	1/1000 inoc.	Heavy inoc.	1/50 inoc.	1/1000 inoc.	Heavy inoc.	1/50. inoc.	1/1000 inoc.	Heavy inoc.	1/50 inoc.	1/1000 inoc.
Controls :												
Without *p*.aminobenzoic acid	+	25	3	+	50	1	+	14	2	+	40 fine	3
1/100,000 *p*.aminobenzoic acid	+*	21	1	+*	50	1	+*	8	2	+*	50–60 fine	1
1/1,000,000 *p*.aminobenzoic acid	+*	30	1	+*	50	*Nil*	+*	9	1	+*	48	1
1/10,000,000 *p*.aminobenzoic acid	+*	33	2	+*	60	2	+*	12	1	+*	54	1
Sulphanilamide 1/5000	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	+ fine	20–30 fine	2 ?
p.hydroxylaminobenzenesulphonamide 1/5000	*c.* 100 fine	1 and 10 v. fine	„	20	„	„	„	„	„	+	20	*Nil*
Sulphanilamide 1/5000 + *p*.aminobenzoic acid 1/100,000	+*	24	„	+*	40	1	+*	7	„	+*	40	3
p.NHOH compound 1/5000 + *p*.aminobenzoic acid 1/100,000	+*	28	„	+*	25	*Nil*	+*	12	„	+	21	1
Sulphanilamide 1/5000 + *p*.aminobenzoic acid 1/1,000,000	+*	24	„	+*	36	„	+*	12	„	+*	40	1
p.NHOH compound 1/5000 + *p*.aminobenzoic acid 1/1,000,000	+	25	1	+	45	„	+	18	„	+ v. fine	12	*Nil*
Sulphanilamide 1/5000 + *p*.aminobenzoic acid 1/10,000,000	+*	28	1	+*	43	1	+*	10	„	+	30	„
p.NHOH compound 1/5000 + *p*.aminobenzoic acid 1/10,000,000	+	32	2	+	30	*Nil*	+	*c.* 10	„	+ fine	25	1

p.NHOH compound = *p*.hydroxylaminobenzenesulphonamide. +* = slightly more than hundreds. + = hundreds.

The above experiment included two more series with 1/10,000 and 1/20,000 dilutions of sulphanilamide and *p*.hydroxylaminobenzenesulphonamide respectively. The readings below are given as they would be taken for the 1/5000 sulphanilamide and *p*.hydroxylaminobenzenesulphonamide series only.

Strain.	Reversal ratio.	*Nil* to complete restoration.	Restricted growth to complete restoration.	*Nil* to partial restoration.
Pneumococcus 2	Sulphanilamide / *p*.aminobenzoic acid	1–>2000	..	..
	p.hydroxylaminobenzenesulphonamide / *p*.aminobenzoic acid	..	1–2000 (1–100*)	..
Pneumococcus 18	Sulphanilamide / *p*.aminobenzoic acid	1–>2000	..	..
	p.hydroxylaminobenzenesulphonamide / *p*.aminobenzoic acid	..	1–100	1–>2000
Pneumococcus 3501	Sulphanilamide / *p*.aminobenzoic acid	1–>2000	..	..
	p.hydroxylaminobenzenesulphonamide / *p*.aminobenzoic acid	1–100	..	1–>2000
Pneumococcus 1834	No reading; degree of inhibition of growth by sulphanilamide and *p*.hydroxylaminobenzenesulphonamide too slight.			

* Reading of column for 1/50 inoculation is taken, but 1–100 seemed the best approximation taking both heavy and 1/50 inoculation into account.

sulphanilamide 1 : 5000 ; for sulphaguanidine 1 : 1000 ; for sulphapyridine 1 : 40 ; for sulphathiazole 1 : 8 ; and for sulphadiazine 1 : 8.

Wood (1942) observed that over a range of concentrations of various sulphonamides in a simple dextrose/asparagine medium for *B. coli* the ratio of the minimum concentration of *p*.aminobenzoic acid needed to prevent bacteriostasis to that of the sulphonamide was constant, and he found the following *p*.aminobenzoic acid/drug average ratios : Sulphanilamide 1 : 1613 ; sulphaguanidine 1 : 1000 ; 4 : 4′-diaminodiphenylsulphone 1 : 1388 ; sulphapyridine 1 : 100 ; sulphadiazine 1 : 100 and sulphathiazole 1 : 36.

The facts that sulphanilamide action is more easily reversed by *p*.aminobenzoic acid than that of sulphapyridine, which in turn is more easily reversed than that of sulphathiazole, and that the bacteriostatic powers are in the order sulphathiazole > sulphapyridine > sulphanilamide, have been shown by several workers in addition to those already cited—Spink and Jermsta (1941), Landy and Dickens (1942), Wiedling (1942), Strauss, Dingle and Finland (1941), and Kohn and Harris (1941).

This extensive literature with regard to the *p*.aminobenzoic acid suppression of the bacteriostatic activities of sulphanilamide all refers to work carried out in fluid media, and much of it involves only the restoration of diminished growth. Apart from some experiments on *B. coli* which have approximated to the more usual technique in observations of this kind, the method adopted in our work has been to prepare a series of tubes of 10 per cent. inactivated ox serum (56°–58° C. for one hour) agar in which we have incorporated various concentrations of sulphanilamide or *p*.hydroxylaminobenzenesulphonamide, and further similar series containing one or more concentrations of *p*.aminobenzoic acid. These mixtures have been poured to plates and inoculated with a heavy inoculum of the bacterium under test and also with one or two dilutions of the first inoculum. The control plates without sulphanilamide or *p*.aminobenzoic acid and with *p*.aminobenzoic acid only have indicated the number of viable bacteria, and a reasonably accurate comparison of one experiment with another has been possible. The full scheme of one such experiment is set out in Table II to illustrate the basis of comparison afforded. The work has been done with the following bacteria : the meningococcus, the pneumococcus, the *C. diphtheriae* and *B. coli*. Some observations with gonococcus relate only to suppression of sulphanilamide effect. In the case of the first three the considerable *in vitro* bactericidal effect of both substances has made it possible to determine the ratio of *p*.aminobenzoic acid to sulphanilamide or to *p*.hydroxylaminobenzenesulphonamide giving the following bases of comparison :

(1) Complete restoration of growth to standard of control where complete sterilization had occurred with sulphanilamide or *p*.hydroxylaminobenzenesulphonamide.

(2) Complete restoration of control standard of growth where considerable inhibition had been observed with sulphanilamide or *p*.hydroxylaminobenzenesulphonamide.

(3) Partial restoration of growth where complete sterilization had occurred with sulphanilamide or *p*.hydroxylaminobenzenesulphonamide. The second of these ratios corresponds more nearly to most of those recorded in the literature. A number of experiments, of course, did not give results from which all these ratios could be calculated and some only permitted of approximate calculation of the ratios, as for example when the lowest concentration of *p*.aminobenzoic acid used still restored growth completely. The significant results are recorded in the Tables III and IV.

As regards the efficacy of *p*.aminobenzoic acid in suppressing sulphonamide bactericidal effect, it is clear that there are striking differences amongst these bacteria. Any very exact numerical evaluation of the figures is obviously unwarranted, but, roughly speaking, the effect of *p*.aminobenzoic acid is about ten times greater for pneumococcus than for the *C. diphtheriae*, and for the latter about ten times greater

TABLE III.—*Suppression of Sulphanilamide Effect by* p.*Aminobenzoic Acid.*

Date of experiment.	Bacterium.	Ratios of $\frac{\text{p.aminobenzoic acid}}{\text{sulphanilamide}}$ giving (*a*) Complete restoration of absent growth.	(*b*) Complete restoration of restricted growth.	(*c*) Partial restoration of absent growth.
27.vii.40	Pneumococcus (a)	..	..	1–100
,,	,, (b)	..	..	1–100
6.viii.40	,, (a)	1–100	..	1–1,000
,,	,, (b)	1–1,000	..	1–100,000
13.viii.43	,, (c)	..	1–10,000	..
,,	,, (d)	1–10,000	1–20,000	1–100,000
15.viii.43	,, (d)	1–10,000	1–20,000	..
10.xi.43	,, (d)	1–2,000	..	..
,,	,, (c)	1–2,000	1–2,000	..
,,	,, (f)	1–2,000	..	..
,,	,, (g)	..	1–2,000	..
24.vii.40	Meningococcus (a)	..	..	1–5
,,	,, (b)	..	..	.. (1–1)*
27.vii.40	,, (b)	..	..	.. (1–1)*
,,	,, (a)	..	..	1–100
31.vii.40	,, (b)	..	..	.. (1–10)*
,,	,, (a)	..	..	1–100
12.viii.43	,, (c)	..	..	.. (1–100)*
,,	,, (d)	..	..	1–1,000
20.xi.43	,, (d)	1–50	1–50	1–500
27.xi.43	,, (c)	1–8	1–200	1–200
,,	,, (d)	1–10	1–200	1–500
17.i.44	,, (I)	..	1–10	1–40
,,	,, (Gale)	..	..	.. (1–8)*
7.i.44	,, (Gale)	..	1–2	1–25
,,	,, (I)	..	..	1–20
,,	,, (II)	..	..	1–50
27.vii.40	Gonococcus (a)	..	1–100	..
,,	,, (b)	..	1–100	..
31.vii.40	,, (a)	..	..	1–100
,,	,, (b)	..	..	1–100
20.xi.43	*B. coli* (i)	..	1–1,000	..
2.xii.43	,, (4508)	..	1–1,000	..
6.xii.43	,, (4508)	..	1–1,000	..
17.i.44	,, (Nat. Coll.)	1–1,000	1–1,000	..
20.xi.43	*C. diphtheriae:*			
	mitis	1–100	1–500	..
	intermedius	1–500	1–1,600	..
	gravis	1–50	1–250	1–1,600
31.i.44	*gravis* (G3)	1–200	1–1,000	1–400
	intermedius (112)	1–30	1–200	..
	mitis (M7)	1–100	1–1,000	1–300
	intermedius (140)	1–200	1–1,000	1–200

* Highest ratio of $\frac{\text{p.aminobenzoic acid}}{\text{sulphanilamide}}$ tried which failed to give any restoration as described in the columns (*a*), (*b*) and (*c*).

TABLE IV.—*Suppression of* p.*Hydroxylaminobenzenesulphonamide Effect by* p.*Aminobenzoic Acid.*

Date of experiment.	Bacterium.	Ratios of $\frac{p.\text{hydroxylaminobenzenesulphonamide}}{p.\text{aminobenzoic acid}}$ giving (*a*) Complete restoration of absent growth.	(*b*) Complete restoration of restricted growth.	(*c*) Partial restoration of absent growth.
13.viii.43	Penumococcus (c)	..	1–1,000	1–20,000
,,	,, (d)	1–1,000	1–4,000	1–10,000
14.viii.43	,, (d)	..	..	1–5,000
10.xi.43	,, (d)	..	1–100	..
,,	,, (e)	1–50	1–1,000	1–2,000
,,	,, (f)	1–1,000	..	1–2,000
12.viii.43	Meningococcus (c)	..	..	.. (1–40)*
,,	,, (d)	..	..	.. (1–40)*
24.xi.43	,, (d)	..	1–2	1–10
24.i.44	,, (I)	1–1	..	1–20
,,	,, (Gale)	..	..	1–10
,,	,, (N.P.)	..	..	1–20
22.xi.43	*B. coli* (4508)	..	..	.. (1–125)*
10.i.44	,, (Stock)	..	..	1–5
13.i.44	,, (Nat. Coll.)	..	..	1–2
,,	,, (M.G.S.)	..	1–4	1–15
,,	,, (Stock)	..	..	.. (1–1)†
,,	,, (175)	..	..	1 (1–1)†
20.xi.43	*C. diphtheriae*:			
	mitis	..	1–250	I–5,000
	intermedius	..	..	1–1,600
	gravis	..	1–5	1–1,600
22.i.44	*mitis*	..	..	.. (1–1)†
	gravis	..	1–40	..
	intermedius	..	..	1–20
	P.W.8	..	..	1–10
31.i.44	*gravis* (G.3)	..	1–20	..
	intermedius (I.12)	1–20	1–100	..
	mitis (M.7)	..	1–40	..
	intermedius (1.140)	1–4	1–100	1–30

* Highest ratio of $\frac{p.\text{aminobenzoic acid}}{\text{sulphanilamide}}$ tried which failed to give any restoration as described in the columns (*a*), (*b*) and (*c*).

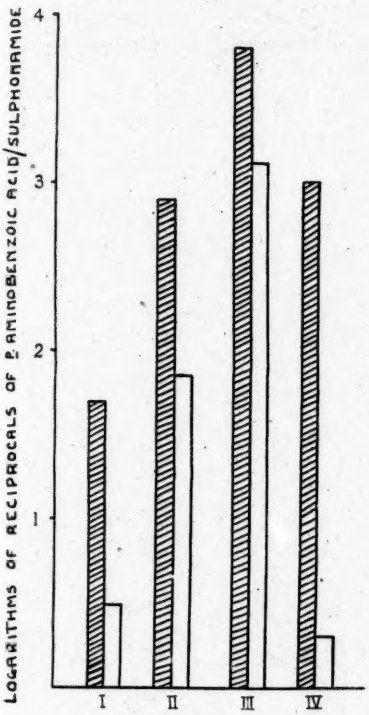
† In these cases there was considerable restoration of partially inhibited growth at the 1–1 ratio.

than for the gonococcus and some strains of meningococcus. There are, however, other strains of meningococcus which are scarcely protected from sulphanilamide at all by *p*.aminobenzoic acid.

Green and Bielschowsky conclude that *p*.hydroxylaminobenzenesulphonamide is unlikely to play a part in sulphonamide action because its bactericidal effect is not neutralized by *p*.aminobenzoic acid. This conclusion is no longer found to be valid when a wider range of bacteria than that examined by them is brought under survey. The neutralization by *p*.aminobenzoic acid is quite marked in the case of the pneumococcus, and in a general way it varies for each of these micro-organisms much in the

Comparison of the Effect of p.*Aminobenzoic Acid in Reversing the Bactericidal Action of Sulphanilamide and* p.*Hydroxylaminobenzenesulphonamide respectively as Observed with Various Bacteria.*

FIG. 1.



I. *Meningococcus.*—Sulphanilamide sensitivity very high. *p*.aminobenzoic acid reversal poor. Ratio:

$$\frac{\text{p.aminobenzoic acid reversal of sulphanilamide}}{\text{p.aminobenzoic acid reversal of p.hydroxylaminobenzenesde.}} = 17$$

II. *C. Diphtheriæ.*—Sulphanilamide sensitivity high. *p*.aminobenzoic acid reversal good. Ratio:

$$\frac{\text{p.aminobenzoic acid reversal of sulphanilamide}}{\text{p.aminobenzoic acid reversal of p.hydroxylaminobenzenesde.}} = 11$$

III. *Pneumococcus.*—Sulphanilamide sensitivity moderately high. *p*.aminobenzoic acid reversal very good. Ratio:

$$\frac{\text{p.aminobenzoic acid reversal of sulphanilamide}}{\text{p.aminobenzoic acid reversal of p.hydroxylaminobenzenesde.}} = 5$$

IV. *B. coli.*—Sulphanilamide sensitivity low. *p*.aminobenzoic acid reversal good. Ratio:

$$\frac{\text{p.aminobenzoic acid reversal of sulphanilamide}}{\text{p.aminobenzoic acid reversal of p.hydroxylaminobenzenesde.}} = 500$$

▨ = *p*.aminobenzoic acid–sulphanilamide reversal ratio.

□ = *p*.aminobenzoic acid–*p*.hydroxylaminobenzene sulphonamide reversal ratio.

In vitro sulphanilamide sensitivity I much > II > III much > IV.

The titres for sulphanilamide and *p*.hydroxylaminobenzenesulphonamide reversal on which this figure is based were determined by taking the average of the figures in column (*b*) in Tables III and IV + the average of one-fifth the figure in column (*c*) where no result was obtained under (*b*). This procedure was adopted since the complete restoration of restricted growth corresponds most nearly to the other observations described in the literature, and because where figures were available in both columns (*b*) and (*c*) on a rough average those in column (*c*) were five times greater. Where failure of reversal was recorded at a low titre, this was taken into account in determining the average.

same way as with sulphanilamide, with the difference that it is about ten times weaker. If, however, one compares *p*.aminobenzoic acid neutralization of sulphanilamide in its action on meningococcus with the corresponding neutralization of *p*.hydroxylaminobenzenesulphonamide effect on the pneumococcus, the latter is the more striking phenomenon.

As far as *B. coli* is concerned we found that working in the fluid synthetic medium of Strauss, Dingle and Finland (1941) we were able to get full restoration of growth of *B. coli* to control standard by using *p*.aminobenzoic acid in the ratio of 1–8000 or 1–10,000, a result which falls very nearly into line with the results recorded in the literature cited above and with our pneumococcus results using the technique described above. When, however, strains of *B. coli* were examined by the technique used with the bacteria for which results are recorded in Tables III and IV, i.e. inoculation of progressive dilutions to agar plates containing sulphanilamide or *p*.hydroxylaminobenzene sulphonamide with *p*.aminobenzoic acid, it became clear that this technique was one which showed the *p*.aminobenzoic acid effect less, probably because sulphanilamide effect is better developed at a surface (Burton, McLeod, Mayr-Harting and Walker, 1942). Under these conditions restoration of growth completely inhibited by sulphanilamide was only obtained with one of the four strains tested, although complete restoration of growth partly inhibited was observed with all, the ratio of concentration of *p*.aminobenzoic acid to sulphanilamide being of the order 1–1000.

Taking, however, only the three bacteria markedly sensitive to sulphanilamide *in vitro* into account, for which a number of observations have been made in parallel conditions, we find, as is indicated in Fig. 1, in which the observations recorded in the tables have been roughly summarized and are compared with similar observations for *B. coli* :

(*a*) That the more sensitive the micro-organism is to sulphanilamide *in vitro* the less effective is *p*.aminobenzoic acid found to be in eliminating that effect.

(*b*) That where the *p*.aminobenzoic acid neutralization of sulphanilamide effect is marked there is also considerable neutralization of *p*.hydroxylaminobenzenesulphonamide effect and vice versa.

SUMMARY AND CONCLUSIONS.

The general conception emerges from these observations that the capacity of *p*.aminobenzoic acid to neutralize sulphanilamide bacteriostasis decreases in proportion to the *in vitro* sensitiveness of the bacterium under examination. If this conception is to be reconciled with the theory of Woods and Fildes it must be assumed that for some strains of meningococcus at any rate the hypothetical enzyme combining with *p*.aminobenzoic acid shows an even higher affinity for sulphonamide. The argument advanced by Green and Bielschowsky, that transformation of sulphanilamide to *p*.hydroxylaminobenzenesulphonamide is unlikely to be necessary to the development of sulphanilamide activity, is not found to be firmly grounded when a wider range of bacteria than those examined by them is brought under investigation, and a technique used in which bacteria grow exposed to air at a surface.

The observation of Green and Bielschowsky that the effect of *p*.hydroxylaminobenzenesulphonamide on *B. coli* is not reversed is mainly confirmed, such reversal as has been observed being of a trifling nature. The correlation of high *in vitro* sensitivity to sulphanilamide with reversal of *p*.hydroxylaminobenzenesulphonamide effect by *p*.aminobenzoic acid is an interesting phenomenon, for which there is at present no very obvious explanation, *p*.aminobenzoic acid reversal of sulphanilamide effect being 1000 times greater than that of *p*.hydroxylaminobenzenesulphonamide in the

case of *B. coli*, but only about ten times greater in the case of the three types of bacteria showing high *in vitro* sensitivity to sulphanilamide.

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REFERENCES.

BURTON, H.—(1941) *Chem. and Industry*, **60**, 449.
Idem, MCLEOD, J. W., MCLEOD, T. S., AND MAYR-HARTING, ANNA.—(1940) *Brit. J. exp. Path.*, **21**, 288.
BURTON, H., MCLEOD, J. W., MAYR-HARTING, ANNA, AND WALKER, N.—(1942) *J. Path. Bact.*, **54**, 407.
BURTON, H., AND WALKER, N.—(1943) *J. Chem. Soc.*, 656.
FILDES, P.—(1940) *Lancet*, i, 955.
GREEN, H. N.—(1940) *Brit. J. exp. Path.*, **21**, 38.
Idem AND BIELSCHOWSKY, F.—(1942), *Ibid.*, **23**, 13.
KOHN, H. I., AND HARRIS, J. S.—(1941) *J. Pharm. and exp. Therap.*, **73**, 343.
LAMPEN, J. O., AND PETERSON, W. H.—(1941) *J. Amer. chem. Soc.*, **63**, 2283.
LANDY, M., AND DICKENS, D. M.—(1942) *Nature*, **149**, 244.
MAYER, R. L.—(1937) *Biol. méd.* (Supplement), **27**, 45, 74.
PARK, C. R., AND WOOD, W. B.—(1942) *Bull. Johns Hopkins Hosp.*, **70**, 19.
RANTZ, L. A. (1942) *Proc. Soc. exp. Biol. Med.*, **49**, 137.
ROSE, H. M., AND FOX, C. L.—(1942) *Science*, **95**, 412.
RUBBO, S. D., AND GILLESPIE, J. M.—(1940) *Nature*, **146**, 838.
SEVAG, M. G.—(1943) *J. Amer. chem. Soc.*, **65**, 110.
SPINK, W. W., AND JERMSTA, J.—(1941) *Proc. Soc. exp. Biol. Med.*, **47**, 395.
STAMP, T. C.—(1939) *Lancet*, ii, 10.
STRAUSS, E., LOWELL, F. C., AND FINLAND, M.—(1941) *J. clin. Invest.*, **20**, 189.
STRAUSS, E., DINGLE, J. H., AND FINLAND, M.—(1941) *J. Immunol.*, **42**, 313.
WIEDLING, S.—(1942) *Nature*, **150**, 290.
WOOD, W. B. (jun.)—(1942) *J. exp. Med.*, **75**, 369.
WOODS, D. D.—(1940) *Brit. J. exp. Path.*, **21**, 74.
WYSS, O.—(1941) *Proc. Soc. exp. Biol. Med.*, **48**, 122.

THE EFFECT OF CELL GROWTH-PROMOTING TISSUE EXTRACTS ON THE HEALING OF EXPERIMENTAL CUTANEOUS WOUNDS IN RATS. I. LOCAL APPLICATION.*

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IN previous communications from this laboratory (Doljanski and Hoffman, 1939; Hoffman and Doljanski, 1939; Doljanski, Hoffman and Tenenbaum, 1939; Hoffman, Tenenbaum and Doljanski, 1940) it was shown that extracts of adult tissue possess pronounced cell growth-promoting power. The growth-promoting activity of adult tissue extracts was found to be completely independent of the state of proliferation of the tissue from which they are made (Hoffman, Tenenbaum and Doljanski, 1939‡). Extracts of certain adult organs—heart, smooth muscle, and brain—activate, under given experimental conditions, the growth of fibroblast colonies *in vitro* to a greater extent than embryonic extract of the same concentration. The stimulating action of adult tissue extract in cell growth is not transient. It is possible to cultivate cells indefinitely in a medium which is composed exclusively of adult plasma and extract of adult heart or adult brain (Doljanski and Hoffman, 1943). The growth-promoting property of extracts of adult tissues is not species-specific. The growth of human skin epithelium can, for instance, be activated by extract of adult fowl heart to a high degree (Doljanski, Hoffman and Tenenbaum, 1942). The active principle in the growth-promoting tissue extracts is probably a protein or a protein-linked group. When saline extracts of adult tissue are precipitated with alcohol, material is obtained which is partly soluble in Ringer or in Tyrode solution; the test in tissue cultures shows that the growth-promoting activity displayed by the alcohol precipitate dissolved in Tyrode solution is not inferior to that of the native extract (Werner and Doljanski, 1942).

In numerous investigations it was claimed that local application of cell growth-promoting embryonic extract accelerates the process of wound healing in man and animals. The extracts of certain adult tissues being more powerful in stimulating cell proliferation *in vitro* than embryonic extract, and moreover, much easier to obtain than the latter, the use of growth-activating adult tissue extracts in wound healing was suggested (Doljanski, Hoffman and Tenenbaum, 1942). A trial of the effect of the cell growth stimulating adult tissue extracts on the process of healing of wounds under properly controlled conditions of animal experiments appeared to be desirable.

In the present paper the question is examined whether adult tissue extracts and

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† Working under the Hadassah Medical Organization fellowship.
‡ See also Trowell and Willmer (1939).

cell growth-promoting preparations therefrom are capable, applied locally, of accelerating the process of healing of experimental wounds; the rate of healing of treated wounds has been compared with that of untreated wounds in the same animals.

MATERIAL AND METHODS.

A. *Preparation of Cell Growth-promoting Tissue Extracts.*

1. *Adult tissue extracts.*—Extracts of heart muscle of adult chicken were used in this study. The hearts were finely minced in a Latapie apparatus, suspended in five parts of Ringer solution and shaken for an hour in an agitator. The suspension was allowed to stand overnight on ice, and was then sharply centrifuged. The supernatant fluid was decanted off and kept in the refrigerator. For use the extracts were incorporated in the ointment base (lanolin anhydrous) in a proportion 40 : 60.

2. *Alcohol precipitates of adult tissue extracts.*—Minced heart muscle of adult fowl was extracted with five parts of Ringer solution. The extract was precipitated with four volumes of 96 per cent. alcohol. The precipitate was centrifuged and then dried over calcium chloride *in vacuo*. The dry product was extracted with ether and ground to a fine powder. The powder was used as such, or incorporated in an ointment base.

3. *Embryonic extract.*—The experiments were carried out with—

(*a*) Undiluted embryonic extract incorporated in an ointment base in a proportion 40 : 60.

(*b*) Embryonic extract diluted with Tyrode solution in a proportion 1 : 3.

The extract was prepared from seven days old chicken embryos. The embryos were removed under sterile conditions, washed in Ringer solution, and passed through a Latapie apparatus. After standing for one hour in the refrigerator the tissue pulp is centrifuged for ten minutes and the supernatant fluid removed.

B. *Technique of Wound-healing Experiments.*

The experiments were performed on white rats weighing from 150 to 200 g. The animals were kept on a standard and well-balanced diet. Two circular wounds, about 20 mm. in diameter, were produced on the back of the animals symmetrically on either side of the dorsal midline at the shoulders. The outlines were first marked out on the skin by means of a cylindrical blade and the skin within the demarked area was then excised with sharp scissors down to the superficial fascia. Ether anaesthesia was used.

Ointments were applied to the wounds in a uniform layer, and the wound was covered with a gauze dressing. The control wound was covered with the same ointment base without addition of tissue extracts. The wounds were re-dressed every two days.

Powder was sprinkled on the surface of the wounds directly, and the wound covered with gauze and protective dressing. Powder was renewed every two days.

In experiments with *fluid extracts* the wounds were covered with gauze, saturated in the case of the experimental wounds with tissue extracts, and in the case of the control wounds with Ringer solution. Both wounds were then covered with a protective dressing. The extracts and Ringer solution were renewed daily.

C. *Measurement of Wound Area.*

The wounds were outlined at each change of the dressing, i.e. daily or on every second day, and the wound area tracings were measured. The values thus obtained were plotted in curves. Anaesthesia (ether) was employed at each dressing to

facilitate more accurate tracings. The criteria of healing were the disappearance of all granulation-tissue, and the complete epithelialization of the wound.

EXPERIMENTAL.

A. *Observations on the Course of Healing in Untreated Wounds.*

The process of repair in untreated wounds may be described as follows : Diminution of the wound area can generally be observed about 24 hours after excision ; it is due to contraction of the wounds' margins. Although experimental and control wounds are made equal in size and symmetrically in position, considerable differences in form and size may result in the first phase of the healing process. These differences gradually disappear as the process of wound healing advances, and two symmetrically placed, equally large wounds generally heal in about the same time. Granulation-tissue appears in the majority of cases on the second day after skin excision ; on the fourth day the wound is almost always covered with well-developed granulations. Between the fifth and seventh day the granulation-tissue reaches the level of the skin, and may even protrude above. On the seventh to ninth day the gross appearance of the granulation-tissue undergoes a change. Whereas previously its colour was fresh, dark red and its texture granular, it now becomes brownish-red and appears to be covered by a homogeneous fine membrane (" upper protoplasmic layer " of Loeb). Epithelialization generally becomes visible between the sixth and eighth day. Wounds of 20 mm. diameter become closed on an average in 22 days, the period of healing varying between 17 and 28 days. The healing time in a series of untreated standard wounds is summarized in Table I.

B. *Observations on the Course of Healing in Treated Wounds.*

1. *Wounds treated with saline extract of adult chicken hearts.*—A total of 10 wounds in 10 animals were treated with ointment containing 40 per cent. of saline extract of adult chicken hearts. The times of healing of the treated wounds and of corresponding untreated controls are summarized in Table II. The total duration of the wound-healing process in the treated wounds was 19·1 days ; in the untreated wounds 17·6 days. Extracts of adult hearts when applied locally did not, thus, accelerate the course of healing of treated wounds as compared with control wounds in the same animal ; they would even appear to cause a slight inhibition.

2. *Wounds treated with alcohol precipitates of extract of adult chicken hearts.* (a) *Dry powder.*—A total of 22 wounds in 22 animals were treated with powdered alcohol precipitates of saline extract of adult chicken hearts. The results of these experiments are summarized in Table III. The time of closure was practically the same in the treated and in the untreated wounds—18·9 days for treated wounds, and 18·5 days for untreated ones.

(b) *Ointment.*—The results of wound treatments with alcohol precipitates of saline extract of adult chicken hearts applied in an ointment form are set forth in Table IV. The closure time was 17·1 days for treated, and 15·4 days for untreated wounds.

There is no significant difference between untreated wounds and wounds treated with saline heart extracts and alcohol precipitates thereof neither in the course of the wound-healing process as a whole, nor in the duration of its individual phases (contraction, formation of granulation-tissue, epithelialization). The gross appearance of the treated wound does not present any unusual features throughout the whole healing process. It may be noted that in wounds which receive ointment treatment, whether

Table I.

Exp. No.	Healing time in days. Left.	Right
50	26	26
52	28	20
54	17	24
56	19	27
58	20	19
60	23	23
62	22	22
64	27	25
66	18	18
68	23	18
70	23	24
79	22	25
80	20	20
81	17	22
82	24	24
Mean	21·9	22·5

Table II.

Exp. No.	Healing time in days. Exp.	Contr.
61	16	20
63	20	13
65	20	16
67	20	18
69	19	18
71	16	16
75	19	19
76	22	21
77	20	16
78	19	19
Mean	19·1	17·6

Table III.

Exp. No.	Healing time in days. Exp.	Contr.
1	16	18
2	19	19
4	25	25
6	16	17
7	20	14
8	15	18
9	15	19
11	26	28
13	15	26
16	20	20
18	16	15
20	23	16
21	26	18
22	25	21
40	18	18
41	19	22
42	16	15
43	17	15
44	16	13
45	18	16
46	20	20
48	15	15
Mean	18·9	18·5

Table IV.

Exp. No.	Healing time in days. Exp.	Contr.
24	18	18
26	16	16
27	18	22
28	16	16
30	14	15
31	17	14
32	17	16
33	17	14
36	14	14
37	18	14
49	24	13
51	20	14
53	14	14
55	16	16
Mean	17·1	15·4

TABLE V.

Exp. No.	Healing time in days. Exp.	Contr.
102	29	16
103	19	16
106	24	20
109	17	20
112	17	18
115	25	19
118	27	18
120	20	16
121	24	14
122	29	18
152	27	18
153	21	23
154	24	17
155	21	17
156	20	15
157	27	22
158	21	19
162	27	24
163	21	17
174	22	22
Mean	23·1	18·5

TABLE VI.

Exp. No.	Healing time in days. Exp.	Contr.
164	16	18
165	15	19
166	20	17
167	19	17
168	22	21
169	20	17
170	17	17
171	20	19
172	21	15
173	20	19
180	19	16
181	20	19
Mean	19·1	17·8

or not the latter contains tissue extracts, epithelialization becomes visible two days earlier on an average than is the case in wounds not covered with ointments.

3. *Wounds treated with saline embryonic extracts.*—In view of the fact that extracts of adult tissue prove incapable of accelerating the rate of healing of treated wounds as compared with untreated wounds in the same animal, it seemed to us of interest to re-examine the findings of earlier investigators who claimed that embryonic extract is effective in accelerating wound healing. The experiments were performed with embryonic extract in liquid form and with embryonic extract incorporated in an ointment base.

(a) *Embryonic extract in liquid form.*—A total of 20 wounds in 20 animals were treated. The results are summarized in Table V. The average rate of repair is 18·5 days in the untreated, and 23·1 days in the treated wounds.

(b) *Embryonic extract incorporated in an ointment base.*—A total of 12 wounds in 12 animals were treated. The closure time of the treated wounds and of corresponding untreated wounds is shown in Table VI. The mean value is 17·8 days in untreated, and 19·1 days in treated wounds.

The extract of embryos, both in liquid and in ointment form applied locally, did not hasten the healing of treated wounds as compared with controls in the same animals. The extract in liquid form even causes a distinct retardation of the healing process.

Day-to-day charts of the treated and untreated wounds showed that there was no difference in the rate of healing at any period.

DISCUSSION.

Attempts to accelerate the process of wound healing by local application of cell growth-promoting material are of early date. In his first communication on the activation of cell growth in cultures *in vitro* by tissue extracts, Carrel (1913) states that in processes of reparation of small cutaneous wounds " the proliferation of the epithelium and of connective tissues was activated by dressings made with the pulp of tissues and organs." This observation attracted wide interest, and, during the last two decades, several papers on the effect of growth-promoting tissue extracts on wound healing appeared. These investigations concerned both indolent human wounds and experimental wounds in animals. In the present paper only the latter will be reviewed.

Wallich (1926) reported a favourable effect of chicken embryonic extract on the healing of experimental skin wounds in guinea-pigs. His paper gives no data of the healing time nor of the number of animals used. The embryonic extract employed by this investigator had been passed through an ultra-filter. According to Carrel (1913), such treatment deprives embryonic extract of its cell growth-promoting power.

Bugliari (1927) treated skin wounds in three rabbits (using a fourth as control) with chick embryonic extract applied locally. On the sixth day the treated wounds were reduced in size by 47·4 per cent., and the control wounds by 31·7 per cent. ; on the twelfth day the reduction was 73·5 per cent. and 51·3 per cent. respectively.

Nakamura (1930) treated skin wounds of guinea-pigs with favourable results. He failed to give quantitative data on the rates of repair, nor does he mention the number of animals used. He did not use original extract, but globuline fractions of the latter. Unfortunately he presented no details as to the mode of preparation of these fractions, and gave no information on their cell growth-promoting power *in vitro*.

Amorosi (1931) treated skin wounds in guinea-pigs with extracts of ten days old rabbit embryos. The local application of the embryonic extract was carried out in five animals, while another five served as controls. After four days there was a reduction in size of the wounds on an average of 14 per cent. in the control wounds and of 30 per cent. in the experimental wounds ; after eight days of 24 per cent. in the control wounds and of 50 per cent. in the experimental wounds ; after twelve days of 32 per cent. in the control wounds and of 80 per cent. in the experimental wounds.

McJunkin and Matsui (1931) undertook experiments to determine the effect of macerated dead homologous tissue on the healing of experimental skin wounds in rats. The following tissues were used : cornified epithelium, foetal epidermis, liver, and tail tendon. The authors concluded that macerated homologous epidermis applied to wounds stimulates epidermal regeneration ; the proliferative activity of the epidermis is not much affected by macerated liver ; the macerated connective-tissue retards wound healing.

Morosov and Striganova (1934) carried out wound-healing experiments on 62 rats. The wounds were treated with saline extracts of 4–6 weeks old human embryos. They noted a positive effect of embryonic extract both on the formation of granulation-tissue and on the process of epithelialization. The activation of the granulation-tissue was stronger than that of the epithelialization. The acceleration of wound healing was about 12 per cent. on an average. These authors also used dried granulation-tissue in their studies. The effect of the latter preparation was inferior to that of embryonic extract.

Nielsen (1939) reported wound-healing experiments carried out with four dogs. He noted favourable effects with local application of three different kaolin adsorbate preparations from calf foetus extracts. The average acceleration obtained was about 33 per cent. The methods of preparation of the adsorbates were not indicated.

The above positive results are in contrast to the findings of Dvorak and Byram and of Dann, Glücksmann and Tansley.

Dvorak and Byram (1930) treated experimental skin wounds in 14 dogs by applying the pulp of the following organs to their surfaces : foetal liver, foetal kidney, spleen, muscle, and skin of different animals and man. " No tissue investigated caused any striking acceleration of healing." It should be noted, however, that the growth-activating power *in vitro* of the material tested by the authors is either unknown or, as has been shown recently (Hoffman, Tenenbaum and Doljanski, 1940), it is small.

Dann, Glücksmann and Tansley (1941) investigated the effect of " Epicutan "* on superficial and deep wounds and burns in 64 rats. They showed that Epicutan does not " greatly affect the rate of decrease in the wound area nor the rate of the epithelialization." On the other hand they noted marked improvement of collagen and muscle regeneration. It must be emphasized in this connection that, according to Fischer, Epicutan stimulates the growth of tissue cultures *in vitro* ; tissue culture experiments by Willmer have, however, shown that *in vitro* Epicutan stimulates the migration, but not the division of cells (*cf.* Dann, Glücksmann and Tansley, 1941).

Most of the hitherto performed wound-healing experiments were carried out with material whose cell growth-promoting activity *in vitro* is either questionable or unknown. The experiments carried out with preparations whose cell growth-promoting activity *in vitro* is certain are, on the other hand, in most cases inadequately supported by numerical data. Their evaluation requires therefore great caution. For the same reasons comparison of the previous findings with those reported above is hardly feasible.

The results of the investigation presented in this paper may be summarized as follows :

Extracts of adult chicken heart and alcohol precipitates of such extracts which possess great cell growth-activating power *in vitro* do not, when applied locally, promote the healing rate of treated wounds as compared with control wounds in the same animals. Cell growth-promoting extracts of embryonic tissues have also no accelerating effect. The course of all phases of the wound-healing process (contraction, formation of granulation-tissue, epithelialization) are the same in treated as in control wounds.

The experiments which have been reported here deal exclusively with the effect of cell growth-promoting tissue extracts on the rate of repair of *healthy* experimental wounds. The question of the efficacy of the use of cell growth-activating extracts in the case of *indolent* wounds remains open and is under investigation.

SUMMARY.

1. The saline extract of adult chicken heart and the alcohol precipitate of this extract, as well as saline extract of seven days old chicken embryos, were applied locally to experimental skin wounds in rats.

2. No improvement of the rate of repair could be obtained with the preparations used. The process of healing was not hastened in treated wounds as compared with control wounds in the same animals.

* Calf embryonic extract, absorbed on kaolin (manufactured by Messrs. Lundbeck, Copenhagen).

REFERENCES.

AMOROSI, O.—(1931) *Riv. Patol. sper.*, **6**, 33.
BUGLIARI, G. R.—(1927) *Ann. ital. Chir.*, **6**, 779.
CARREL, A.—(1913) *J. exp. Med.*, **17**, 14.
DANN, L., GLÜCKSMANN, A., and TANSLEY, K.—(1941) *Brit. J. exp. Path.*, **22**, 70.
DOLJANSKI, L., AND HOFFMAN, R.—(1939) *C. R. Soc. Biol.* (*Paris*), **130**, 1246.
Idem.—(1943) *Growth*, **7**, 67.
Idem AND TENENBAUM, E.—(1939) *C. R. Soc. Biol.* (*Paris*), **131**, 432.
Idem.—(1942) *Nature*, **150**, 23.
DVORAK, M. J., AND BYRAM, J. W.—(1930) *Proc. Soc. exp. Biol.* (*N.Y.*), **27**, 967.
HOFFMAN, R., AND DOLJANSKI, L.—(1939) *Growth*, **3**, 61.
HOFFMAN, R., TENENBAUM, E., AND DOLJANSKI, L.—(1939) *Nature*, **143**, 764.
Idem.—(1940) *Growth*, **4**, 207.
MCJUNKIN, F. A., AND MATSUI, T.—(1931) *Arch. Path.*, **12**, 794.
MOROSOV, D. B., AND STRIGANOVA, A. R.—(1934) *J. Physiol. Path. gén.*, **32**, 1148.
NAKAMURA, T.—(1930) *C. R. Soc. Biol.* (*Paris*), **104**, 191.
NIELSEN, E.—(1939) *Ugeskr. Laeg.*, **37**, 1071.
TROWELL, O. A., AND WILLMER, E. N.—(1939) *J. exp. Biol.*, **16**, 60.
WALLICH, R.—(1926) *C. R. Soc. Biol.* (*Paris*), **95**, 1480.
WERNER, H., AND DOLJANSKI, L.—(1942) *Nature*, **150**, 660.

EFFECTS OF SOUTH WALES ANTHRACITE COAL AND OF PRECIPITATED AMORPHOUS SILICA UPON THE LUNGS OF MICE.

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In a previous paper (Campbell, 1943) some reference was made to preliminary experiments in which mice inhaled dust from anthracite and bituminous coals obtained from the South Wales coal-mines, where the miners have been troubled with lung symptoms. These coal-dust experiments were undertaken in 1940 on the suggestion of Dr. P. D'Arcy Hart (1942), who was engaged with other workers on an inquiry into pulmonary disease in certain South Wales coal-mines for the Committee on Industrial Pulmonary Disease under the Medical Research Council. The coals used in my experiments were obtained through the courtesy of Dr. Donald Hicks, of the South Wales Coal Survey Laboratory, Cardiff.

My dusting experiments with mice were undertaken in the first place about eight years earlier in connection with the effects of tarred-road and other dusts upon the incidence of lung tumours in mice. The influence of coal dust upon this incidence is considered herein, together with the effects of combination with either silica or alumina. Also, together with the effects of inhalation of coal dust upon the lung fibrous tissue, the additional influences of alcohol, up to 5 per cent. in the drinking-water, and of a moderate degree of cigarette smoke in the atmosphere, have been examined. In addition, the influence of aluminium and of alumina upon formation of silicotic nodules in the tracheobronchial lymph-nodes of the mouse has been investigated.

Technique.

As a rule 150 mice about three months old were used in each experiment, 75 for exposure to the dust and 75 for the control. If, at an early stage in the experiment, an epidemic occurred, additional mice were added so that eventually about 40 to 60 mice, aged ten months or older, would be obtained. Equality of numbers for sexes and for colour of mouse was maintained at the start of each experiment. Each mouse box contained about 8–10 mice, all of the same sex, to avoid fighting. A cloud of dust was passed into the respiration chamber at regular intervals—hourly or half-hourly, etc.—over six hours for five days of each week for a year or more. The temperature of the chamber was usually about 20° C. The mice were transferred to clean boxes with bedding, food and water every evening. Their diet consisted of brown bread moistened with water, bran, oats, hay, some greenstuff, and cod liver oil on two days of each week. As a rule all the mice were allowed to fulfil their allotted span of life, and their organs, especially the lungs, were examined carefully for pathological changes. Sections for microscopic examination were cut and stained either by the

haematoxylin and eosin method, or by the Weigert-van Gieson method, or by Robb-Smith's modification of the Foot and Mènard method for fibrous (reticular) tissue.

Dust counts were made soon after the dust was blown into the chamber, and counts up to 5000 particles per cubic centimetre were obtained with the Owen's dust counter. The sizes of the particles in the dust cloud in the chamber were mostly between 1 and 3μ.

The inorganic substances, precipitated amorphous silica, alumina and aluminium, were obtained from the British Drug Houses. The silica or alumina was added in equal quantity with the coal dust and the aluminium in the proportion of 1 part to 4 parts silica dust. For the heavy dusting with silica, thick clouds of dust were passed into the chamber every 20 minutes.

The cigarette smoke was used in the same way and in the same degree as described for previous experiments (Campbell, 1936). The alcohol was added to the drinking-water at first to form a 1 per cent. solution, and this was maintained for a week or more, when the strength was raised to 2 per cent. By gradual increase the strength was finally raised to 5 per cent. The mice drank this at the rate of about 1 c.c. each *per diem*, but controls drank rather more water than this up to about 2 c.c.

To compare the effects of the presence of the dust in the lung, it is necessary to have a method of estimating the degree of dust deposit in the tissue. This has been done by the microscopical method described in a previous paper (Campbell, 1943), where eight different degrees of dust deposit were distinguished. This method only gives the degree of deposit of coloured dust, such as coal. Silica dust does not give a deposit in the lung capable of estimation in this manner, but since silica produces fibrotic nodules in the tracheobronchial lymph-nodes of the mouse, the intensity of the fibrous tissue formation in these has been used as a measure of the silica dust passing through the lungs. Four types of fibrous tissue increase, " well marked," " marked," " slight," and " very slight," may be distinguished in the lymph-nodes (Campbell, 1942*a*). Examples of " well-marked " increases are shown in illustrations of fibrotic nodules published in a previous paper (figs. 3, 5 and 6, Campbell, 1941).

Incidence of Lung Tumours.

Since lung tumours are very rare indeed in mice younger than ten months, only mice older than this are considered as a rule in this paper. At ten months of age the dusted mice have been exposed to the dust clouds for seven months. The main details of the incidence of lung tumours, simple and malignant, are shown in Table I, and the statistical significance of any increase due to the dusting is considered in Tables II and III by means of the value R. This value R gives the statistical ratio of the difference between the percentage incidences of lung tumours in the dusted mice and in the control mice to the standard error of the difference. When the value of the ratio in column 2 of Table II is 2, it means that such a difference in tumour incidence would arise by chance only once in 20 trials ; if the ratio is 2·5, the difference would arise by chance only once in 80 trials, and if 3, only once in 370 trials. The increases in lung tumours are considered to be of definite significance if the value of R is 2·5 or greater. With R value of 2 up to 2·5 the increase is of only border-line significance. With a value of R below 2 the increase may have been due to chance. In only two of the present experiments is the increase in incidence of lung tumours due to the dust of definite significance. These two experiments are dusting with a mixture of anthracite coal and alumina where the value R = 2·8, and dusting with a mixture of silica and alumina where R = 2·6. These increases in incidence are probably due to the silica and the alumina and not to the anthracite coal, since the other experiments with

anthracite coal do not give any significant increases. In previous experiments silica has given significant increases (Campbell, 1943). Some increase was also obtained with cigarette smoke in previous experiments (Campbell, 1936), and this may explain in part the increase of border-line significance observed in Experiment 3*b*, Table II. Alumina and aluminium seem to have some activity in promoting tumours in combination with silica (Experiments 2 and 3*a*, Table II). An experiment (No. 12*a*, Table II) with alumina alone gave no increase in incidence of lung tumours, but the lung tissue appeared to contain very little dust; apparently the dusting was not heavy enough, so that the mice were able to get rid of the dust by way of the trachea or by other means. In a similar experiment with magnesia (No. 12*b*, Table II) the lungs showed no increase of dust over that in the controls, so that the magnesia had been removed by way of the trachea or by absorption into the tissues.

TABLE I.—*Total Numbers and Percentages (in brackets) of Mice, Living* 10 *Months or Longer, with Lung Tumours.*

Experiment in order of ratio R.	Animals with lung tumours.					
	Control.			Dusted.		
	Total.	Simple.	Malignant.	Total.	Simple.	Malignant.
1. Anthracite + Al_2O_3	1 (2·3)	1 (2·3)	0 (0·0)	9 (20·9)	7 (16·3)	2 (4·6)
2. SiO_2 + Al_2O_3	1 (2·3)	1 (2·3)	0 (0·0)	9 (18·0)	7 (14·0)	2 (4·0)
3*a*. SiO_2 + Al	2 (4·2)	2 (4·2)	0 (0·0)	8 (22·2)	4 (11·1)	4 (11·1)
3*b*. Anthracite + alcohol + SiO_2 + cigarette smoke	1 (3·0)	1 (3·0)	0 (0·0)	9 (18·4)	7 (14·3)	2 (4·1)
5. Bituminous coal	1 (1·9)	1 (1·9)	0 (0·0)	8 (12·1)	2 (3·0)	6 (9·1)
6. Anthracite + alcohol + cigarette smoke	1 (3·0)	1 (3·0)	0 (0·0)	7 (14·3)	4 (8·2)	3 (6·1)
7. SiO_2 (heavy)	1 (2·9)	1 (2·9)	0 (0·0)	6 (11·5)	3 (5·7)	3 (5·7)
8. SiO_2 + alcohol	1 (2·2)	1 (2·2)	0 (0·0)	4 (9·5)	2 (4·7)	2 (4·7)
9*a*. Anthracite + alcohol	2 (3·8)	2 (3·8)	0 (0·0)	5 (8·9)	2 (3·6)	3 (5·3)
9*b*. Anthracite + SiO_2	2 (4·2)	1 (2·1)	1 (2·1)	5 (10·2)	2 (4·1)	3 (6·1)
11. Anthracite coal	4 (7·0)	2 (3·5)	2 (3·5)	5 (9·1)	4 (7·3)	1 (1·8)
12*a*. Al_2O_3	2 (3·3)	1 (1·6)	1 (1·6)	1 (1·5)	1 (1·5)	0 (0·0)
12*b*. MgO	2 (4·3)	2 (4·3)	0 (0·0)	1 (1·9)	1 (1·9)	0 (0·0)

TABLE II.—*General Data* re *Duration of Experiments and Numbers of Surviving Mice. Significance of Increase in Incidence of Lung Tumours—Ratio R.*

Ratio (R) numercial order.	Ratio R.	Experiment.	Total number of mice living 10 months or longer.		Age of oldest dusted mice at death.	Period covered by dust in the atmosphere.	Mice alive at 20 months expressed as percentage of those alive at 10 months.	
			Control.	Dusted.	Days.	Days.	Control.	Dusted.
1.	2·8	Anthracite + Al_2O_3	44	43	923	692	41	47
2.	2·6	SiO_2 + Al_2O_3	44	50	879	654	41	50
3*a*.	2·4	SiO_2 + Al	48	36	721	253	50	31
3*b*.	2·4	Anthracite + alcohol + SiO_2 + cigarette smoke	33	49	778	545	27	37
5.	2·1	Bituminous coal	52	66	804	365	13	33
6.	1·9	Anthracite + alcohol + cigarette smoke	33	49	778	575	27	37
7.	1·6	SiO_2 (heavy)	35	52	738	579	30	31
8.	1·5	SiO_2 + alcohol	45	42	879	365	20	24
9*a*.	1·1	Anthracite + alcohol	53	56	813	365	19	39
9*b*.	1·1	Anthracite + SiO_2	48	49	890	630	41	32
11.	0·4	Anthracite coal	57	55	819	365	32	51
12*a*.	*Nil*	Al_2O_3	59	60	815	365	24	16
12*b*.	„	MgO	45	53	826	365	11	33

The alcohol had no effect, in the concentrations employed, upon the incidence of lung tumours. The age of the mice has an important influence upon the incidence of lung tumours (Campbell, 1942*b*), and must be considered in the present cases (Table II, column 6). Further, Table II gives, in columns 8 and 9, the precentages of mice alive at 20 months for both the controls and experimental mice respectively. It is apparent that the increases in the four experiments at the top cannot be due to difference in age between the controls and the dusted mice. The two experiments (Nos. 1 and 2, Table II) with the highest values for R were exposed to the longest periods of dusting, nearly two years, which might explain their relatively high incidence of lung tumours, but in experiment No. 9*b*, Table II, the dusting was nearly as long, yet the incidence of lung tumours shows no significant increase. However, it is not the period of dusting that is the only important factor; there is also the degree of coal-dust deposit in the lung; this is shown in Table III. It is obvious that the degree of deposit in the lungs does not explain the order of the values of R, nor does the degree of silicotic nodule

TABLE III.—*Statistical Significance of Increase in Lung Tumours. Degree of Coal Dust Deposit in Lungs and Lymph-nodes. Fibrotic Nodules (SiO_2). Mice 10 Months or Older.*

Experiment in order of ratio R.	Statistical significance of increase in lung tumour.	Percentage of mice with deposits of coal classified as "moderate to heavy" or higher in lungs.	Percentage of mice with deposits of coal classified as "moderate to heavy" or higher in lymph-nodes.	Percentage of mice with "marked" or "Well marked" SiO_2 nodules in lymph-nodes.
1. Anthracite + Al_2O_3	Definitely significant	60	30	..
2. SiO_2 + Al_2O_3	,, ,,	..	..	50
3*a*. SiO_2 + Al	Border-line significance	..	..	22
3*b*. Anthracite + alcohol + SiO_2 + cigarette smoke	,, ,,	6	47	41
5. Bituminous coal	,, ,,	83	73	..
6. Anthracite + alcohol + cigarette smoke	Increase might be due to chance	92	39	..
7. SiO_2 (heavy)	Ditto	..	..	31
8. SiO_2 + alcohol	,,	..	..	43
9*a*. Anthracite + alcohol	,,	84	55	..
9*b*. Anthracite + SiO_2	,,	39	47	57
11. Anthracite	No significance	25	22	..
12*a*. Al_2O_3	No increase	..	..	..
12*b*. MgO	,,	..	..	..

TABLE IV.—*Number of Tumours per Mouse with a Lung Tumour. Recent Experiments.*

Experiment in order of ratio R.	Average number of lung tumours per mouse with a lung tumour.		Maximum number of lung tumours in any one mouse.	
	Control.	Experiment.	Control.	Experiment.
1. Anthracite + Al_2O_3	1·0	1·2	1	3
2. SiO_2 + Al_2O_3	1·0	1·4	1	3
3*a*. SiO_2 + Al	1·5	1·0	2	1
3*b*. Anthracite + alcohol + SiO_2 + cigarette smoke	1·0	1·4	1	3
5. Bituminous coal	1·0	2·0	1	5
6. Anthracite + alcohol + cigarette smoke	1·0	1·4	1	2
7. SiO_2 (heavy)	3·0	1·7	3	2
8. SiO_2 + alcohol	1·0	1·7	1	3
9*a*. Anthracite + alcohol	1·0	1·7	1	3
9*b*. Anthracite + SiO_2	1·0	1·4	1	2
11. Anthracite coal	1·4	1·7	4	2
12*a*. Alumina	1·5	1·0	2	1
12*b*. Magnesia	1·0	1·0	1	1
Average	1·3	1·4	1·5	2·4

formation in the tracheobronchial lymph-nodes (column 5, Table III), which gives an indication of the amount of silica passing through the lungs. As in previous papers (Campbell, 1943), some unknown factor or factors are playing some part. Susceptibility of certain cells of the alveoli has been suggested previously as the cause of increase of lung tumours in dusting and other experiments. This susceptibility seems to be controlled by heredity. It would appear that the stimulus from dust is more

TABLE V.—*Number of Tumours per Mouse with a Lung Tumour. Previous Experiments.*

Experiment in order of ratio R.	Average number of lung tumours per mouse with a lung tumour.		Maximum number of lung tumours in any one mouse.	
	Control.	Experiment.	Control.	Experiment.
1. Tarred road dust (1)	2·0	3·0	2	7
2. CO + tarred road dust	4·0	1·9	6	4
3. $Al_2O_3 + SiO_2 + Fe_2O_3 + CaCO_3$	0·0	1·8	0	6
4. Fe_2O_3	2·6	1·5	7	4
5. Czechoslovak dust (2)	1·0	1·7	1	4
6. Tar-free road dust	3·0	6·9	9	17
7. Tarred road dust (2)	1·0	1·8	1	4
8*a*. Diluted nickel dust	1·3	2·3	2	7
8*b*. Czechoslovak dust (1)	2·0	1·7	2	3
10. SiO_2 + "nickel" dust	1·5	1·6	2	2
11*a*. $SiO_2 + Fe_2O_3$	2·6	1·7	7	4
11*b*. Carbon exhaust soot	2·9	4·3	6	9
13. $Al_2O_3 + SiO_2 + Fe_2O_3$	1·5	2·5	3	7
14. Steel grindings	1·2	2·5	2	5
15. Coal soot	2·9	1·6	6	3
Average	2·0	2·5	3·7	5·7

TABLE VI.—*Increase of Fibrous (Reticular) Tissue in Lungs of Mice, 10 Months or Older. Coal Experiments.*

Experiment in order of degree of coal deposit.	Percentages of mice with coal deposits in lungs classified as "moderate to heavy" or higher.	Percentages of mice with increase of fibrous tissue in lung classified as—			
		"Very fair" + "fair."	"Very fair."	"Fair."	"Slight."
1. Anthracite + alcohol + cigarette smoke	92	55	24	31	8
2. Anthracite + alcohol	84	34	12	21	12
3. Bituminous coal	83	0	0	0	15
4. Anthracite + Al_2O_3	60	16	0	16	35
5. Anthracite + SiO_2	39	16	2	14	30
6. Anthracite coal	25	0	0	0	7
7. Anthracite and alcohol + SiO_2 + cigarette smoke	6	10	0	10	30

TABLE VII.—*Increase of Fibrous (Reticular) Tissue in Lungs of Mice, 10 Months or Older. Silica Experiments.*

Experiment in order of degree of silicotic nodule formation in tracheobronchial lymph-nodes.	Percentages of mice with silicotic nodules in tracheobronchial lymph-nodes classified as "marked" or "well marked."	Percentages of mice with increases in fibrous tissue in lung classified as—			
		"Very fair" + "fair."	"Very fair."	"Fair."	"Slight."
1. Anthracite + SiO_2	57	16	2	14	30
2. $SiO_2 + Al_2O_3$	50	10	2	8	16
3. SiO_2 + alcohol	43	7	0	7	2
4. Anthracite + alcohol + SiO_2 + cigarette smoke	41	10	0	10	30
5. SiO_2 (heavy)	31	13	2	11	15
6. SiO_2 + Al	22	11	3	8	14

often chemical rather than mechanical in its action on the susceptible cells of the lung. It is obvious that the dusts which are active in increasing the incidence of lung tumours do not cause tumours wherever the dust collects, so that certain areas only are susceptible. This point is illustrated in Table IV, which gives the number of tumours per mouse with a lung tumour in the present series of experiments. As so few of the control mice had lung tumours, a better and fairer comparison perhaps is the maximum number of lung tumours in any one mouse (columns 4 and 5, Table IV). Here, on an average, the dusted mice show an increase over the controls, the figures being 1·5 and 2·4 respectively. Table V gives similar information for previous series of experiments, of which Nos. 1 to 7 gave definitely significant increases in incidence of lung tumours. The average figure for the maximum number of lung tumours in any one experimental mouse is 5·7, compared with 3·7 for the controls. Injection into the veins of some powerful chemical carcinogenic agents recently discovered increases greatly the numbers of lung tumours in some strains of mice, but not in others, showing again a difference in susceptibility (see previous papers for literature, Campbell, 1943). The lung tumours resulting from these carcinogenic agents are usually simple adenomas. Some of the lung tumours resulting from exposure to dusts are very malignant (see figs., Campbell, 1937, 1943), at least as malignant as any produced by the new chemical carcinogenic agents. Usually the dusting experiments which show an increase in incidence of lung tumours show also an increase in the proportion of tumours which are malignant (Table I), and also an increase in size of the tumour. Most of the simple tumours in the present experiments both in the controls and dusted mice were small, less than 3 mm. in diameter. Size of tumour increases with malignancy and age, and eventually a whole lobe may be involved. The largest lung tumour in the dusted mice in the present series of experiments was 8 mm. × 8 mm. (Experiment No. 6, Table I), that in the control mice 4 mm. × 4 mm. (Experiment No. 3*a*, Table I).

Fibrosis in the Lung Tissue and Tracheobronchial Lymph-nodes.

Drs. D'Arcy Hart (1942) and Thomas Belt (1942) with their colleagues have observed an increase in fibrous tissue in the lungs of the South Wales coal-miners, particularly in those exposed to anthracite coal dust, and this dust may produce either a reticular tissue increase or fibrotic nodules somewhat like those typical of silica. An increase in the fibrous (reticular) tissue strands in the lung has been observed in some of the mice in the present experiments with anthracite coal, and to a much less extent with the bituminous coal. For comparative purposes it was possible to distinguish three degrees of increase in fibrous tissue strands in the dusted mice's lungs over that seen in the corresponding lung tissue of the control mice. These three degrees have been classified as " very fair," " fair," and " slight." In no case was the increase in fibrous tissue in the form of masses as seen in the nodules produced by silica in the tracheobronchial lymph-nodes of the mouse (figs. 3, 5 and 6, Campbell, 1941), but sections stained with the Weigert-van Gieson method or with Robb-Smith's modification of the Foot and Mènard method showed the increase in the fibrous (reticular) tissue strands in the lung distinctly in many cases, something like the fibres at the edges of the masses shown in these figs. 3, 5 and 6. The experiments with the heaviest deposits of anthracite coal dust showed the greatest percentage of mice with lungs revealing " very fair " plus " fair " increases of fibrous strands surrounding the lung alveoli (Table VI), and the alcohol and cigarette smoke are not responsible at all. The bituminous coal, although in heavy deposit, only produced slight increases in the fibrous tissue in the lungs. Alumina, aluminium and silica also do not affect the fibrous

tissue in the lung to the same extent as the anthracite coal. In contrast with the condition in the lung, the anthracite coal and bituminous coal produced no increase at all in fibrous tissue in the mouse's tracheobronchial lymph-nodes, although there were often very heavy deposits of coal dust present (column 4, Table III). This difference of effects in the lungs and in the lymph-nodes may be due to the absence of movement in the latter, such as is present during inspiration and expiration in the lung. The resulting " irritation without inflammation " may cause the increase of fibrous tissue in the lung. The anthracite coal, being harder than the bituminous coal, produces the greater effect. Other observers hold that the increase in fibrous tissue in the lung of man is due to some contamination of the carbon of the coal, such as silica or silicates, but, as seen below (Table VII), the silica does not seem responsible in the mouse. In the experiments with silica the increase in fibrous tissue in the lung is less marked on the whole than that in the anthracite coal experiments (Table VI), although the silica often produces (Table VII) marked or well-marked fibrotic nodules in the tracheobronchial lymph-nodes. As in previous experiments (Campbell, 1942) with mice the lung seems to pass on its silica to the lymph-nodes, whereas in man the lung seems to retain the dust (Belt and Ferris, 1942). An attempt was made to fill up the lung tissue with silica by heavy dusting (Experiment No. 7, Tables I, II and III), but the result was a blocking of the alveoli of the lung with catarrhal cells containing silica, and the lung still seemed to pass on any of its absorbed silica to the lymph-nodes, where fibrotic nodules form. The heavy dusting with silica did not produce any great increase in fibrous tissue in the lung (Table VII). It is obvious, therefore, that in the mouse anthracite coal dust produces more effect upon the fibrous tissue of the lung than upon that of the tracheobronchial lymph-nodules, whereas silica does the opposite. Thus, the increase in fibrous tissue in the mouse's lung with the anthracite coal does not seem to be due to any silica the coal contains ; it might be due to silicate. In some of my other mouse experiments, e.g. with iron oxide, there is a similar tendency for an increase in fibrous strands ; this occurs in haematite miners, but silica is usually regarded as responsible.

An important finding is that aluminium and alumina did not inhibit formation of silicotic nodules in the tracheobronchial lymph-nodes of the mouse. The mouse's lymph-nodes appear to differ from the rabbit's lung (Denny *et al.*, 1937). Some guinea-pigs have also been exposed to the coal dusts, and although these experiments are not yet completed, some of the lungs show some increase in fibrous tissue.

Increase of Lymph Tissue and other Pathological Changes.

Tumours in other organs than the lungs were rare. Five of the 779 experimental mice and 2 of the 666 controls possessed mammary tumours. Tapeworm cysts were occasionally found in the liver, which also exhibited atrophy and necrosis of cells in a fair number of cases. These changes have not been fully followed up yet.

As in previous experiments with dusts, the lymph tissue in the lungs and tracheobronchial lymph-nodes more often revealed hyperplasia and reticulosis than did that of the controls (Table VIII). The lymph tissue in other organs of the body was not affected in this way to any extent.

The incidence of pneumonia was also higher in the dusted mice as a rule than in the controls (Table VIII). The heavy dusting with silica had to be curtailed on this account, since silica in large amount seems to cause pneumonic changes rather than increase in fibrotic tissue in the lungs of mice. This has been noted by others (verbal communication).

TABLE VIII.—*Some Pathological Data. Mice All Ages.*

Experiment.	Percentages of mice with mammary tumours.		Percentages of mice with increase of lymph tissue in—						Percentages of mice with pneumonia.	
			Tracheobronchial nodes.		Lungs.		Other organs (general).			
	Control.	Expt.	Control.	Expt.	Control.	Expt.	Control.	Expt.	Control.	Expt.
1. Anthracite + alcohol + cigarette smoke	0·0	1·5	11·9	36·8	4·5	14·7	1·5	2·9	14·9	25·0
2. Anthracite + alcohol	0·0	0·0	19·7	43·4	4·5	7·9	1·5	1·3	15·2	23·7
3. Bituminous coal	0·0	0·0	9·5	52·0	1·3	16·0	0·0	5·3	16·0	18·7
4. Anthracite + Al_2O_3	0·0	1·3	6·8	28·9	5·7	15·8	2·3	1·3	22·7	25·0
5. Anthracite + SiO_2	1·5	1·4	10·8	52·1	7·7	36·6	3·1	1·4	13·8	29·6
6. Anthracite coal	0·0	0·0	17·1	30·7	1·3	8·0	1·3	1·3	11·8	13·3
7. Anthracite + alcohol + SiO_2 + cigarette smoke	0·0	1·3	11·9	40·5	4·5	24·3	1·5	4·0	14·9	22·9
8. SiO_2 + Al_2O_3	0·0	1·2	6·8	40·5	5·7	32·1	2·3	3·5	22·7	36·9
9. SiO_2 + alcohol	0·0	0·0	20·3	45·9	14·5	28·4	2·9	2·7	14·5	28·3
10. SiO_2 (heavy)	0·0	0·0	4·9	37·6	6·2	23·4	0·0	2·1	12·3	31·2
11. SiO_2 + Al	1·5	0·0	3·0	44·3	4·5	24·1	1·5	1·5	18·2	56·8

COMMENT.

Compared with the increase obtained with a tarred road dust (Campbell, 1934) upon the incidence of lung tumours in mice, the increases observed with the present dusts are slight. They agree more closely with some increases obtained in some previous experiments (Campbell, 1943) with silica and with iron oxide, and with a tarred road dust from which the tar had been removed by benzene (Campbell, 1937). In this latter experiment, although the incidence of lung tumours was lower, the malignancy of the tumours was greater than that exhibited with the original tarred road dust. The cause of this increase in malignancy is probably the greater age of the mice in the experiment with the tar removed. When the tar was present cancer of the skin developed also, and this killed the mice sooner. Lung tumours in mice, as stated above, increase in size and malignancy with age. This occurs in the control mice as well as in the dusted mice, but to a much less degree. The types of cells seen in the lung tumours of the control mice are similar to those seen in the dusted mice, but malignancy is much less often observed. This has been demonstrated in the previous publications of the author.

The changes produced by anthracite coal in the fibrous tissue of the mouse's lung resemble the earlier stages of changes in the human lung. As the mouse lives only two to three years, the time is perhaps too short for more marked effects to develop in the lung.

SUMMARY.

1. Anthracite coal dust does not increase the incidence of lung tumours in mice. The statistically significant increases observed in some of the present experiments were due to other substances present—silica, aluminium and alumina. A heavy dusting of the lungs with bituminous dust produces some increase in lung tumour incidence, but only of border-line significance. A moderate amount of alcohol does not affect the incidence of lung tumours.

2. As in man, anthracite dust in itself produces an increase of the fibrous (reticular) tissue strands of the lung tissue of mice, and is more active in this respect than bituminous coal. Neither anthracite nor bituminous coal in heavy deposit increases the fibrous tissue of the mouse's tracheobronchial lymph-nodes. The nodular type of fibrosis seen often in the human lung with anthracite coal is not seen in mice. Perhaps the time—duration of mouse's life—is too short.

3. Silica does not increase the fibrous (reticular) tissue strands of the lung of mice to the same extent as anthracite coal, but it produces marked fibrotic nodules in the tracheobronchial lymph-nodes. Its effects differing from those of anthracite coal make it doubtful whether the increase of fibrous tissue in the mouse's lung produced by anthracite is due to its silica content. It might be due to silicate. Alumina and aluminium do not influence the effects of anthracite coal upon the fibrous tissue of the lung.

4. Neither alcohol in the drinking-water in moderate amount nor inhalation of cigarette smoke have any effect upon the action of anthracite coal on the fibrous tissue of the mouse's lung.

5. Metallic aluminium and alumina do not inhibit the development of fibrotic nodules in the tracheobronchial lymph-nodes of mice. The tissue of these nodes, therefore, reacts differently from the lung tissue of rabbits.

In addition to those mentioned in the text, I am indebted to my technical assistant, Mr. C. Pergande, for help in this research, and to Mr. F. J. Higginson for cutting and staining the sections.

Addenda.

1. The following analysis of the anthracite coal used in these experiments was made in the Government chemist's laboratories:

The ash was prepared and analysed according to the British Standard Methods (B.S. No. 1016–1942). The following results were obtained:

Constituent.	Expressed as—	Per cent.
Silica	SiO_2	27·04
Alumina	Al_2O_3	26·32
Iron oxide	Fe_2O_3	22·78
Calcium oxide	CaO	7·33
Barium oxide	BaO	0·70
Magnesium oxide	MgO	4·18
Sodium oxide	Na_2O	2·47
Potassium oxide	K_2O	0·41
Titanium oxide	TiO_2	0·70
Manganese oxide	Mn_3O_4	0·04
Sulphate	SO_3	6·89
Phosphate	P_2O_5	0·67

Spectrographic examination indicated the presence of traces of lead and nickel and slight traces of tin.

2. Dr. Campbell's death occurred while this paper was in the Press. It seems desirable to place briefly on record the results obtained in guinea-pigs exposed to anthracite dust under the same conditions as the mice. Six guinea-pigs were killed after being exposed over a period of 4 years and five others after 3½ years. All showed extensive deposition of coal-dust in the lungs; one only, one of those dusted for 3½ years, showed in addition a localized adenomatous proliferation of bronchial epithelium, probably attributable to the dusting.

References.

Belt, Thomas H., and Ferris, Allan A.—(1942) *Spec. Rep. Ser. Med. Res. Coun.*, London, **243**, 203.

Campbell, J. Argyll.—(1934) *Brit. J. exp. Path.*, **15**, 287.—(1936) *Ibid.*, **17**, 146.—(1937) *Ibid.*, **18**, 215.—(1941) *Ibid.*, **22**, 76.—(1942*a*) *Brit. Med. J.*, i, 217.—(1942*b*) *Brit. J. exp. Path.*, **23**, 191.—(1943) *Brit. Med. J.*, i, 179.

Denny, J. J., Robson, W. D., and Irwin, D. A.—(1937) *Canad. med. Ass. J.*, **37**, 1.

Hart, P. D'Arcy, and Aslett, E. A.—(1942) *Spec. Rep. Ser. Med. Res. Coun.*, London, **243**, 2.

EXPERIMENTS ON THE INHIBITOR OCCURRING IN ROUS No. 1 SARCOMAS.

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THE relation of the filterable tumours of fowls to other neoplasms has remained an outstanding problem of cancer research, and a satisfactory solution seems no nearer than when the first of these tumours was described by Rous in 1911. In general, the filterable tumours have been spontaneous growths, while fowl tumours induced by chemical agencies have proved non-filterable, though exceptions are well known (it is convenient to retain the conventional terms "filterable" and "non-filterable" to designate agent- or virus-transmitted tumours and those transmissible only by intact cells respectively); this work has been reviewed by Murphy and Sturm (1941). It has often been suggested, more or less explicitly, that the difference between the two types is only one of degree and not of kind, and that the non-filterable tumours are only cases in which the isolation of a filterable agent is for some reason or other of extreme technical difficulty (Gye and Purdy, 1930*a*; Cramer and Foulds, 1930). This suggestion receives support from the many reports of the Rous No. 1 tumour itself being temporarily "non-filterable," the best example being that reported by Gye and Andrewes (1926). It is notable that many of these reports date back to the early days of work upon these tumours, before inactivation of the agent by oxygen was recognized, and the difficult feat of filtering a very viscous extract through highly-absorbing material was attempted, the filtrate being tested by inoculation into birds of dubious age and antecedents. After the demonstration by Ledingham and Gye (1935) that the agent could be separated from the extract and purified to some extent by a process of fractional centrifugation, and the subsequent widespread use of this method in place of filtration, together with the use of anti-oxidants during the processing as first recommended by Gye and Purdy (1930*b*), reports of inactive preparations seem to have become less frequent.

Many workers have demonstrated that some extracts of non-filterable agent-induced tumours will inactivate cell-free Rous agent *in vitro*. Experiments on this "inhibitor" action have been reported by Gye and Purdy (1931), Sittenfield, Johnson and Jobling (1931), Murphy and Sturm (1932*a*; 1932*b*), Fraenkel (1938), and Claude (1939). Many other workers have reported experiments which appear to indicate the existence of this substance, and have stated that further work on the problem will be undertaken, though additional data have not yet materialized. This is probably in part due to the rarity and inconstant appearance of the phenomenon.

During the course of work on the inheritance of resistance to the Rous No. 1 sarcoma in fowls, it was found that a large proportion of the fowls kept for some time after the regression of any tumours produced by the testing inoculation suffered a recurrence of the malignant growth, and that all these tumours were examples of non-filterable

Rous tumours (Carr, 1942). It was also found that slow tumours induced in a resistant line of fowls and in certain other resistant birds were similarly prone to yield non-infective filtrates (Carr, 1943*b*). As described below, all these tumours that were tested showed the "inhibitor" effect. It therefore became necessary to investigate the nature and action of this "inhibitor" to determine what relation it bore to the resistance to the Rous tumour agent exhibited by these birds.

Occurrence of Inhibitor.

First of all, an analysis was made of the infectivity of extracts of agent preparations obtained from a series of Rous tumours of assorted types, from which it was concluded that the infectivity of the tumours varied chiefly with the duration of growth of the tumour in the host; after 40 days' growth active agent could no longer be extracted from the tumour. This aspect of the work has been described in a previous paper (Carr, 1943*b*). This preliminary work much assisted further investigations by indicating which tumours could be expected to contain no extractable agent, and hence a probable high content of inhibitor, without previous testing being necessary. Of nine such tumours that were proved to be non-filterable, all were found to possess a marked inhibitor action.

As an example the experiment carried out on the recurring tumour of fowl O.274 will be described. The history of this bird has already been given (Carr, 1942). The tumour was preserved in glycerol, and the effect of this method of preservation was controlled by a parallel test on a specimen of the non-filterable fowl sarcoma GRCH/15 similarly preserved; it was known that this tumour when fresh yielded an extract devoid of any marked anti-Rous activity, and did not induce the formation of antibodies to Rous No. 1 virus (unpublished).

Experiment carried out 5.xi.42.

1 g. of O.274 tumour taken on 16.xii.41 and 1 g. of GRCH/15 tumour taken on 16.vi.41, each preserved in 50 per cent. glycerol, were extracted with 5 ml. of Ringer solution, and the extracts clarified by centrifuging. The extracts and a fresh specimen of serum from a young chick were mixed with a suspension of Rous agent, incubated for two hours, and then injected as shown in Table I. Each inoculum was 0·5 ml., and contained about 20 minimal infective doses (M.I.Ds.) of agent when mixed.

TABLE I.—*Showing Neutralization of Rous Agent by Extract of a Non-filterable Rous No. 1 Tumour.*

Inoculum.		Site.	Fowl No.		
			618.	619.	620.
Rous agent	+ recurring Rous extract .	Left breast .	—	—	—
,,	+ normal fowl serum .	Right ,, .	+	+	+++
,,	+ GRCH/15 extract .	Left leg .	+	++	+++
,,	+ normal fowl serum .	Right ,, .	+	++	+++

The size of the tumours is indicated by the number of + signs.

Not only recurring tumours, but also those which were non-filterable by virtue of their slow and lasting growth, were similarly shown to contain a quantity of inhibitor.

Serum Antibody in Fowls Bearing Non-filterable Rous Tumours.

It was previously shown (Carr, 1943*c*) that all fowls recovering from Rous No. 1 tumours maintained a high content of agent-neutralizing antibodies in their serum.

As it was now possible to predict which tumours were going to be non-filterable, it became feasible to determine the antibody content of the serum of birds bearing such tumours. In all, seven birds were tested, and all were found to have a high content of neutralizing antibodies in their serum. As an example, an experiment carried out on a non-filterable recurring tumour will be given in detail.

Fowl O.646 was tested for susceptibility to Rous agent on 30.v.41 by the routine method (Carr, 1942), the largest inoculum containing approximately 100 M.I.Ds. of agent. Small tumours appeared in the breasts but soon regressed. No recurrence was noted up to 27.vi.42, but a small tumour was noted in the right breast 7.vii.42, which grew rapidly and killed the bird on 27.vii.42. A blood sample was taken on 9.vii.42, and 0·25 ml. of the serum or a 1 : 5 dilution of it completely inactivated 50 M.I.Ds. of agent contained in an equal volume of suspension. The recurring tumour yielded an inactive extract, though cell-grafts grew readily.

This once again illustrates the fact that serum antibodies cannot protect a fowl against the growth of tumour cells. But it is obvious that an extract of minced tumour from these birds will contain the agent suspended in a fluid which may be regarded as dilute antibody, and inactivation of agent is to be expected. Cell grafts, on the other hand, would be expected to grow normally, as antibody transferred by the graft would have no effect on the cells and would soon be diluted by the tissue fluids of the host and the inactivating action lost. It is thus apparent that some at any rate of the inhibiting action of the extracts that were investigated was due to antibodies. It remained to see whether this was the only inactivating material present. Though the suggestion that antibody and inhibitor are identical is not new, most workers have regarded this as improbable, chiefly because of the experiments of Murphy and Sturm (1932*b*), who claimed that "inhibitor" prevented the growth of certain mammalian tumours, while antibody had no such action. As the experimental evidence was that two out of four specimens of crude "inhibitor" reduced the growth of one out of three types of mouse tumours, while a smaller series of tests on an unstated number of antisera had no such action, this cannot be regarded as decisive proof that the two are distinct entities.

Fractionation of Extract Containing Inhibitor.

Claude (1939) reported experiments indicating that the inhibitor is protein in nature. In confirmation of this, it has been found in one experiment that the inhibiting material could be salted out by ammonium sulphate, and that this product could be dissolved in saline to give a solution with all the inactivating action of the original extract.

Flocculation of Tumour Virus.

Flocculation of fowl tumour agent when mixed with antiserum was first demonstrated by Ledingham and Gye (1935). It is therefore to be expected that if antibody and virus are together in the extract of a non-filterable Rous tumour some flocculation may be expected. This has been a constant feature of the present investigation of these tumours. It has been noted that cell-free extracts of tumours found to be non-filterable are much less stable than extracts of routine tumours, and that addition of extract of non-filterable Rous tumours to an otherwise stable active extract or purified agent suspension will cause flocculation. This indicates that the amount of antibody is relatively large. It is obvious that this formation of floccules will cause loss of agent in the preparation of cell-free extracts whether centrifugation or filtration is employed to prepare the extract, quite apart from the agent-inactivating power of the fluid. It was also noted that preparations of purified "virus" obtained from non-filterable

Rous tumours by carrying out the same procedure as will yield an active suspension of Rous agent from young Rous tumours are unstable in suspension. Though virus and " inhibitor," like virus and antibody, do not form an immediate stable and inactive complex, it is possible that some antibody will remain on the virus particles after separation has been attempted, thus causing a tendency to flocculate without causing complete inactivation. It was found that a stable active suspension of Rous virus became similarly unstable when recovered in an active form from an inactive mixture of virus and antibody by centrifuging. In passing, it might be noted that a similar action appeared to be a cause of complications in agglutination tests carried out on Rous virus suspensions. It was noted that suspensions made from old tumours were less stable and more sensitive to antibody than those made from young tumours by the same method. The difficulty of getting comparable results was so great that this line of research was abandoned.

The most complete examination of this flocculation was made on the tumour of O.646 referred to above, processed simultaneously with a routine 24-day-old Rous tumour; these preparations, or the sera obtained from the corresponding birds, are referred to as N-F and F respectively in Table II. All agent suspensions and serum dilutions were made in saline containing 0·2 per cent. formalin, and equal volumes of each were used in the test. Readings were taken after incubating at 37° overnight.

TABLE II.—*Showing Flocculation of Virus by Non-filterable Rous Tumour Preparations.*

Suspension tested.		Testing material diluted	1 : 1.	1 : 2.	1 : 4.	1 : 8.	0.
Purified N-F virus	+	N-F serum	++	++	++	+	+
,, ,, ,,	+	F ,,	+	+	+	+	..
,, F ,,	+	N-F ,,	++	++	++	..	—
,, ,, ,,	+	F ,,	?	—	—	..	..
,, ,, ,,	+	N-F extract	+++	+++	++	..	..
,, ,, ,,	+	F ,,	±	—	—	..	..
Crude N-F extract	+	N-F serum	+++	+++	++	++	+

The degree of flocculation is shown by the number of + signs.

The pH of all tubes was tested at the end of the experiment and found to be the same.

Gye and Purdy (1930*b*) reported that tumour extracts and extracts of normal tissues will sometimes turn cloudy on incubating for prolonged periods, and ascribed this to the action of an oxidase, noting that HCN, which prevents the oxidative destruction of Rous virus, will also inhibit the formation of the cloudiness. They noted, however, that the development of the cloudiness and the amount of destruction of the virus did not always run parallel, and that it was sometimes necessary to add HCN in concentrations which they had previously found to cause destruction of the virus in order to prevent the formation of the cloudiness. In the experiments described above, the action of enzymes would be prevented by the formalin present. It may therefore be concluded that the opalescence noted in tumour extracts may sometimes be due to antigen-antibody combination in addition to the factors such as oxidases which operate in extracts of normal tissues.

Absorption of Inhibitor.

In the case of a tumour whose activity has been reduced but not abolished by inhibitor action, it is to be expected that most of the antibody will be absorbed on the

virus particles and discarded when the cell-free extract is prepared by centrifuging. There is no reason to suppose that "inhibitor" will be similarly lost. This point was investigated in one experiment. A 28-day-old tumour was found to have its activity reduced to 5×10^4 agent particles per gramme of tissue, presumably by the action of inhibitor (see Carr, 1943*b*, for data on the increasing activity of inhibitor with age of tumour). A 10 per cent. cell-free extract of the tumour was heated to 56° for 1½ hours to destroy the activity of agent without harming "inhibitor" (Murphy and Sturm, 1932*a*), but was found to have no effect on a purified Rous agent suspension. This demonstrates that the inhibiting material responsible for the reduced activity of the tumour was all lost in the preparation of the cell-free extract, presumably by the formation of floccules as described above. The similarity of this experiment to an ordinary antibody absorption experiment is obvious. This suggests that the whole of the "inhibitor" can be removed by absorption on tumour agent material, and suggests that only antibody was the cause of the reduced activity of this tumour.

Origin of Inhibiting Material.

Antibody formation is due only to the host. Though it is possible that a tumour may produce antibody, the question does not arise in the present circumstances for, in the first place, cell grafts of these non-filterable tumours did not continue to produce inhibitor (see Carr, 1942), and, secondly, this would not account for the production of inhibitor only in old tumours (Carr, 1943*b*). The following experiment was designed to demonstrate that all the action of the inhibitor encountered in this work can be ascribed to the host antibodies alone.

The chemically-induced non-filterable sarcoma GRCH/15 originated by Peacock was known to produce an extract which had no inactivating action on the Rous agent *in vitro* when the tumour was grown in young fowls (one experiment proving this is shown in Table I). It was also known that this tumour would grow in many birds immune to the Rous sarcoma (Carr, 1943*a*). Grafts of this tumour were made into the following four birds :

Fowl 658 : A bird taken at random from the Institute flock, whose agent-induced Rous tumours produced by inoculation 8.iii.43 had regressed.

Fowl 665 : Similar to the previous bird, agent inoculation performed 10.iii.43.

Fowl O.625 : Recovered from inoculation of tumour material made on 30.v.41. A high content of antibody to Rous agent was known to be always present in the serum.

Fowl O.1273 : Rous tumours regressed after inoculation made 13.viii.41. Not used for any other experiments.

All four birds were successfully grafted with GRCH/15 tumour cells on 13.v.43. As it has previously been shown (Carr, 1943*c*) that all birds after recovery from Rous tumours have a permanent high serum antibody content, antibody will be present in the GRCH/15 tumours in amounts that will be comparable with the amount in Rous tumours growing in similar birds. If anything, the GRCH/15 tumour, being more compact and non-haemorrhagic, will contain a lesser amount than would a Rous No. 1 tumour. Tumour material was taken from fowl 658 when killed 16.vi.43, from O.625 at death (due to visceral gout) 23.vi.43, and was removed by operation from the remaining two birds the same day. All tumours were microscopically and macroscopically entirely GRCH/15 in type. A 10 per cent. extract of each tumour was made, and tested for anti-virus activity by mixing with an equal volume of Rous agent suspension and inoculating into groups of susceptible chicks. It was found that the extract of fowl 658 completely inactivated 1000 M.I.Ds. of Rous agent, and

extracts of the remaining three tumours completely inactivated 100 M.I.Ds. All four extracts were devoid of tumour-producing activity when tested alone.

This demonstrates that the " inhibitor " action depends upon the host, and not on the tumour, and it is clear that the amount of antibody present in a tumour can be sufficient to inactivate all the agent that can be extracted from the tumour cells.

DISCUSSION.

In the present investigations into non-filterable Rous No. 1 tumours it has been shown that all birds bearing such tumours possessed a high antibody content in their serum, and that this alone is sufficient to inactivate free Rous virus. Also that the " inhibitor " causes flocculation of virus and can be absorbed from an extract by union with the virus—properties characteristic of antibodies. The antibody causes loss of activity in two ways, both by formation of floccules in the extract, with loss of agent in the preparation of the cell-free extract, and by inactivation of the remaining virus. It is apparent that these two mechanisms must have been responsible for some part of the activity ascribed to " inhibitor " by other workers. Though in this investigation it was found that this antibody alone was sufficient to produce all the inactivation of virus necessary to produce inactive tumour extracts, this does not rule out the presence of an additional " inhibitor " in the non-filterable tumours investigated by others. Nevertheless, many of the properties of the " inhibitor " reported in the literature are consistent with the view that it was this immunity action that was investigated. Thus Claude (1939) concluded that " inhibitor " was a protein destroyed by enzyme action. An increase in the activity of certain Rous tumour extracts had previously been found after enzyme action by Baker and McIntosh (1927), and by Fränkel and Mislowitzer (1930). Murphy and Sturm (1932*a*) found that " inhibitor " was inactivated at about the same temperature as Rous antibody, and that it could be removed by successive extraction of tumour material (*cf.* separation of virus and antibody by dilution shown by Andrewes, 1932). The separation of " inhibitor " and virus by centrifuging is paralleled by the separation of antibody and virus shown by Amies and Carr (1939). There are, however, two observations not in agreement with this conclusion. The experiments of Murphy and Sturm (1932*b*) on the effect of " inhibitor" on mammalian tumours are discussed above. Fränkel (1938) reported that " inhibitor" was extracted by lipoid solvents. This finding is contrary to the conclusions of all others who have worked on this subject, and the data that he offers in support of this conclusion is by no means decisive ; a confirmation of this work has not yet appeared.

The primary non-filterable Rous tumours became non-filterable because their slow growth enabled the host to produce an effective antibody concentration ; there is no reason to assume that this " inhibitor " action was a cause of their slow growth, as it is admitted that " inhibitor " action and rate of growth are not necessarily related (Murphy and Sturm, 1932*b* ; Carr, 1943*b*). The recurring Rous tumours owe their non-filterability to the antibody produced by the latent agent (Carr, 1943*c*). The time at which the " inhibitor " action becomes apparent, and the time at which it reaches an effective level (Carr, 1943*b*), are in agreement with the time expected for antibody production.

From this work on the Rous No. 1 sarcoma it can be concluded that a similar " inhibition " would also be found in the case of other virus-induced tumours. This is, of course, well known to be true of other fowl tumours, and a similar conclusion that antibody was responsible for the " inhibitor " of the Shope papilloma was reached by Friedewald (1939, 1940). It is therefore pertinent to call into question the majority

of experiments which have been performed to demonstrate the non-existence of infective viruses in tumours. The production of antibody alone may cause a sharp fall in the virus content of extracts of old tumours similar to that found for the Rous No. 1 (Carr, 1943*b*), and experiments on old tumours are thus invalid. Nor can it be agreed that any method of processing will eliminate the " inhibitor " effect if loss of virus by flocculation is not taken into account (*cf.* Murphy and Sturm, 1940).

Throughout this work only tumours from hosts in reasonably good health were used. This is an important restriction, as it is conceivable that severe ill-health of the host will cause a reduction in the amount of circulating antibody sufficient to allow a tumour to return to the filterable state. Such conditions are especially liable to occur with the more slowly-growing tumours. Furthermore, the expression " non-filterable," as applied in this work to the Rous No. 1 sarcoma, indicated that an extract failed to cause a tumour within three weeks when injected into susceptible birds, but workers who use more slowly-growing avian tumours often keep their animals under observation for many months. It is not always clearly recognized that this double standard of assessing filterability exists ; yet the difference is important, as some preliminary experiments (unpublished) have shown that Rous No. 1 tumour extracts that are non-filterable by the usual standard may yet cause tumours in a proportion of inoculated fowls after a delay of many months.

SUMMARY.

Serum antibody to the Rous No. 1 virus was always associated with the presence of " inhibitor " action, and is considered to be identical with it. The antibody causes a reduction in the amount of virus obtained from tumour extracts by forming floccules of virus and antibody which are lost in the preparation of cell-free extracts, and by neutralization of virus remaining in suspension. The amount of antibody contained in a tumour growing in an immune bird is sufficient to inactivate all the virus that could be extracted from the tumour cells. No evidence was obtained that a non-filterable tumour retained this property on transplantation into a non-immune host.

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REFERENCES.

Amies, C. R., and Carr, J. G.—(1939) *J. Path. Bact.*, **49**, 497.
Andrewes, C. H.—(1932) *Ibid.*, **35**, 243.
Baker, S. L., and McIntosh, J.—(1927) *Brit. J. exp. Path.*, **7**, 257.
Carr, J. G.—(1942) *Ibid.*, **23**, 206.—(1943*a*) **24**, 127 ; (*b*) **24**, 133 ; (*c*) **24**, 138.
Claude, A.—(1939) *Amer. J. Cancer*, **37**, 59.
Cramer, W., and Foulds, L.—(1930) *9th Sci. Rep. Cancer Res. Fd., London.*
Fraenkel, E. M.—(1938) *Tumori*, **12**, 114.
Idem and Mislowitzer, E.—(1930) *Z. Krebsforsch.*, **31**, 178.
Friedewald, W. F.—(1939) *Proc. Soc. exp. Biol., N.Y.*, **42**, 330.—(1940) *J. exp. Med.*, **72**, 175.
Gye, W. E., and Andrewes, C. H.—(1926) *Brit. J. exp. Path.*, **7**, 81.
Idem and Purdy, W. J.—(1930*a*) *Ibid.*, **8**, 212 ; (*b*) 282.—(1931) ' The Cause of Cancer.' London (Cassell).
Ledingham, J. C. G., and Gye, W. E.—(1935) *Lancet*, i, 376.
Murphy, J. B., and Sturm, E.—(1932) *J. exp. Med.*, **56**, (*a*) 107 ; (*b*) 483.—(1941) *Cancer Research*, **1**, 609.
Rous, P.—(1911) *J. exp. Med.*, **13**, 397.
Sittenfield, M. J., Johnson, B., and Jobling, J. W.—(1931) *Amer. J. Cancer*, **15**, 2275.

THE ACTION OF SULPHONAMIDES AGAINST *TREPONEMA RECURRENTIS.*

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THIS paper describes an investigation of the prophylactic action of sulphapyridine and other sulphonamide compounds in suppressing infections of mice due to *Treponema recurrentis*. The literature contains three previous reports on this subject. Feldt (1941) used mice infected intraperitoneally with a sensitive strain of *Sp.* (*Trep.*) *usbekistanica*. On the day after infection he administered orally or subcutaneously two doses of sulphapyridine or sulphathiazole, 10 mg. per 20 g. mouse, and found that the spirochaetes disappeared from the blood within 24 hours. Other strains responded to doses of 20 mg. per 20 g., but spirochaetes persisted in the brain. Other sulphonamide compounds were ineffective, and substitution in the pyridine or thiazole rings, or introduction of other heterocyclic rings or other organic groups into the sulphapyridine molecule, led to loss of activity. The action of sulphapyridine and sulphathiazole was not inhibited by *p*-aminobenzoic acid. Ishii, Shimizu and Tsuda (1941) worked with a strain of *Treponema duttoni* and the compounds were given by mouth, one dose daily for four days beginning on the day of inoculation. Sulphapyridine was effective in suppressing the infection when given in doses of not less than 10 mg. per mouse of about 13 g. No activity was discovered in the other sulphonamide compounds examined, viz. sulphanilamide, Diseptal A (4-(*p*-aminobenzene sulphonamide)-benzene sulphondimethylamide) and sulphamethylthiazole. On the other hand, Vargas and Zozoya (1941) found that sulphadiazine, sulphapyridine and sulphanilamide, 45 mg. daily, did not affect the course of the infection in mice.

METHODS.

The parasite used was a strain of *Trep. recurrentis* obtained from the Liverpool School of Tropical Medicine, through the kindness of Dr. J. D. Fulton. Inoculations were made intraperitoneally, each mouse receiving 0·2 c.c. of a suspension of spirochaetes, such that there were 1–4 spirochaetes present in each field of an ordinary coverslip preparation when examined with a dark-ground illumination microscope. Preliminary experiments were made in which infected mice were dosed with sulphapyridine by intraperitoneal injection or by other routes. Some therapeutic activity was observed but the results were irregular, and in subsequent experiments the drug was administered by mixing it in the ordinary wet mash diet of the mice.

The mice were allowed to feed for several hours on this diet before infection. The resultant blood concentration was measured by killing one or more mice after 24 hours or more, withdrawing blood from the heart and estimating the concentration in the usual way by Bratton and Marshall's technique. In early experiments it was found that blood taken about 5 p.m., after the mice had been feeding all day, tended to

contain higher concentrations than that taken in the morning before new food was given; but the difference was not great in view of the large individual variation between different mice. In later experiments the blood for estimation was usually taken about 5 p.m. The blood of the mice was examined each day as a coverslip preparation on a dark-ground illumination microscope.

The results of a typical experiment are given in Table I, which shows the course of the infection in groups each of 4 mice placed on normal and on sulphapyridine diets respectively. In the control mice the infection reaches its maximum about the third day and then spontaneously declines, the blood becoming free from spirochaetes in six or seven days. In the mice on a diet containing sulphapyridine, infection is usually either partially or completely suppressed. In most experiments, suppression was more complete than that exhibited in the table, which was chosen to illustrate more clearly the mathematical treatment of the results. In order to summarize the data and to express them in a quasi-quantitative fashion more suitable for comparison, the following arbitrary procedure was adopted:

Infections of less than 1 spirochaete per microscope field were given a value of 2, those of 1 to 20 spirochaetes a value of 3, those of more than 20, i.e. + and ++, a value of 4, and those with no spirochaetes a value of 0. The figures for the groups of mice on each day were added up and divided by the number of mice alive on that day, the figures obtained being shown in the line designated "Summary" in Table I. The total of these figures for the first four days of the experiment was taken to indicate the degree of infection in that group of mice. The activity of the drug was expressed as—

$$\frac{\text{(4-day total for controls)—(4-day total for treated mice)}}{\text{(4-day total for controls)}} \times 100$$

as is illustrated in Table I. If the infection in the treated mice was heavier than in

TABLE I.—*The Effect of Sulphapyridine in Suppressing Spirochaetal Infections in Mice.*

	Infection.					Total of summary for first 4 days.
Days:	1.	2.	3.	4.	5.	
Controls	—	+	+	3/1	2/1	
	1/5	15/1	+	2/1	—	
	—	2/1	+	+	1/1	
	1/3	3/1	++	+	D	
Summary	1	3·25	4	3·5	2	11·75
Sulphapyridine diet (1·5 per cent.)	—	4/1	—	—	—	
	2/15	8/1	+	++	D	
	—	—	—	—	—	
	—	—	—	—	—	
Summary	0·5	1·5	1	1	0	4·0

$$\text{Activity of sulphapyridine} = \frac{11{\cdot}75 - 4{\cdot}0}{11{\cdot}75} \times 100 = 66 \text{ per cent.}$$

The blood concentration of sulphapyridine in an additional mouse on diet, killed at 5 p.m., was 14 mg. free and 16 mg. total per 100 c.c.

The figures 1/5, 15/1, etc., under infection indicate the number of spirochaetes per microscope field (oil-immersion); + and ++ indicate many and very many respectively. For "summary" and other figures see text.

the controls, this expression gives a negative value. The results obtained in individual experiments varied somewhat in an irregular manner and too much emphasis must not be placed on minor differences. Thus, in eleven experiments with sulphapyridine, the values obtained for the activity were 74, 66, 19, 93, 100, 100, 88, 47, 100, 100, 70. Roughly it may be said that average values below 20 per cent. have probably no significance, while average values above 60 per cent. indicate considerable activity (which is, however, much less extensive or constant than that of actively spirochaeticidal compounds such as neoarsphenamine.)

RESULTS.

The results from a long series of experiments are summarized in Table II. The concentration of the different compounds in the diet was approximately the maximum tolerated concentration.

From this table it may be concluded that :

(1) The action of sulphapyridine is not antagonized by *p*-aminobenzoic acid (P.A.B.), by nicotinamide, or by a combination of these two substances. The difference between the activity indices for the various combinations is too small to be significant. Accordingly it seems clear that the antispirochaetal activity of sulphapyridine (and of sulphathiazole) is not an activity of the sulphonamide type, nor is it associated with the metabolism of nicotinamide.

(2) Sulphathiazole is the only other sulphonamide compound (among those tested) which possesses this anti-spirochaetal activity. The failure of such actively bacteriostatic compounds as sulphadiazine effectively to suppress the spirochaetes is particularly significant. Some of the other sulphonamides, e.g. sulphacetamide, sulphadiazine and sulphaguanidine, have average values of between 10–20 per cent., but it is doubtful whether these indicate any real activity ; if present it is slight.

(3) Other compounds containing a pyridine ring did not possess this anti-spirochaetal activity.

Experiments with *Trypanosoma equiperdum* and *Tryp. congolense* with this technique failed to reveal any antitrypanosomal activity of sulphapyridine and other sulphonamides.

DISCUSSION.

The results of the present experiments confirm and extend the conclusions of previous workers reviewed above, viz. that sulphapyridine and sulphathiazole exert an antispirochaetal action. Since this action is not antagonized by *p*-aminobenzoic acid, it is apparently not an action of the sulphonamide type. Similarly it is not antagonized by nicotinamide. When dysentery bacilli are maintained in a synthetic medium, their respiration is stimulated by nicotinamide ; this stimulation is inhibited by sulphapyridine and sulphathiazole, but not by other sulphonamides, and the inhibition due to sulphapyridine cannot be suppressed by *p*-aminobenzoic acid (Dorfman and Koser, 1942). There is no evidence for such a nicotinamide-sulphapyridine antagonism in the present circumstances.

The chemical properties of the thiazole ring are much like those of the pyridine ring, and consequently it is not surprising that the antispirochaetal action of sulphapyridine is also exerted by sulphathiazole. On the other hand, sulphonamides, not possessing one of these heterocyclic rings, do not act in this manner. Apparently the action depends on the pyridine or thiazole ring rather than upon the sulphonamido

TABLE II.—*The Anti-spirochaetal Activity of Miscellaneous Compounds.*

Compound.	Conc. in diet %.	No. of exps.	Prophylactic activity. Range.	Prophylactic activity. Average.	Blood conc. mg. per 100 c.c. Range.	Blood conc. mg. per 100 c.c. Average.
Sulphapyridine	1·5–2	12	19 to 100	80	7·5/10 – 43/43	23/25
Sulphapyridine plus P.A.B.	1·5–2 + 0·1–0·8	9	15 to 100	67	1·2/1·9 – 57/65	18/25
Sulphapyridine plus nicotinamide	1·5–2 + ·02–1	5	51 to 100	77	—	19/19
Sulphapyridine plus P.A.B.+nicotinamide	1·5–2 +0·4 +0·2–1	3	65 to 100	87		
Other Sulphonamide Compounds.						
Sulphathiazole	1–2	3	76 to 100	85	3·3/3·7 – 26/26	11/13
Sulphanilamide	4	2	—21 to 0	—10	—	14/19
Sulphadiazine	1-2	5	—3 to 33	17	28/40 – 34/62	32/49
Sulphamezathine	1·5	3	—8 to 20	2	2·3/2·3 – 51/52	21/25
Sulphacetamide	2	3	0 to 32	16	7·5/12 – 46/46	24/29
Sulphaguanidine	2	3	3 to 33	13	6·8/6·8 – 16/18	10/12
Irgamid	2	1	—	0	—	—
Prontosil rubrum	2	1	—	6	—	11/26
Diaminodiphenyl-sulphone	0·1–0·3	3	—25 to —13	—18		
Compounds Related to Sulphapyridine.						
Acetylsulphapyridine	2–2·4	2	—23 to —3	—13	2·8 – 4·9 / 4·9 – 6·6	2·5 / 5·0
Pyridine 3-sulphonamide	·25–1	4	—21 to 74	23	—	—
Amino pyridine sulphate	1	2	—16 to —13	—15		

The figures in the column for blood concentration indicate the concentration of "free" sulphonamide/the concentration of "total" sulphonamide.

groups, but attempts to produce it with compounds such as acetyl-sulphapyridine, pyridine-3-sulphonamide, or amino-pyridine sulphate, were unsuccessful.

This antispirochaetal action of sulphapyridine and sulphathiazole is not strong enough to possess any practical importance, but it illustrates a new type of chemotherapeutic activity, the significance of which is not yet known.

SUMMARY.

1. If mice infected with *Treponema recurrentis* are fed on a diet containing sulphapyridine (1·5 per cent.), the spirochaetal infection is completely suppressed, or its intensity is much diminished.

2. A similar activity is shown by sulphathiazole, but not by the other sulphonamides, nor by certain compounds containing the pyridine group, e.g. pyridine 3-sulphonamide, aminopyridine sulphate, or acetyl sulphapyridine.

3. The antispirochaetal action is not antagonized by *p*-aminobenzoic acid, nor by nicotinamide.

4. Apparently the action is not of the sulphonamide type; it probably depends upon the pyridine or thiazole rings rather than upon the *p*-aminobenzene sulphonamide groups.

5. The action is not great enough to possess clinical importance, but it illustrates a new type of chemotherapeutic activity.

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REFERENCES.

Dorfman, A., and Koser, S. A.—(1942) *J. infect. Dis.*, **71**, 241.

Feldt, A.—(1941) *Klin. Wschr.*, **20**, 945.

Ishii, N., Shimizu, S., and Tsuda, K.—(1941) *Jap. J. exp. Med.*, **19**, 5.

Vargas, L., and Zozoya, J.—(1941) *Rev. Inst. Salubridad Enfermedades Trop.* (Mexico), **2**, 303; *Trop. Dis. Bull.*, **39**, 693.

THE LIBERATION OF VIRUS, TOGETHER WITH MATERIALS THAT INHIBIT ITS PRECIPITATION WITH ANTISERUM, FROM THE SOLID LEAF RESIDUES OF TOMATO PLANTS SUFFERING FROM BUSHY STUNT.

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IN all previous work on the properties of plant viruses the starting material has been the sap that is expressed from macerated tissues by squeezing through cloth. Leaves or whole plants have usually been macerated by passage through a domestic meat mincer or by pounding them with a pestle in a mortar. Most workers have frozen the leaves, the mince or the sap, as this facilitates clarification and purification but it does not increase the amount of virus extracted. Washing the leaf residues yields little more virus, usually only the amount to be expected from the volume of sap contained by the residues. It has therefore been assumed that the leaf tissues contain no significant amount of virus, and the published figures for the virus content of infected plants refer solely to the amounts extracted in the sap.

This assumption is unjustified, for we have found that the leaf tissues hold virus in considerable quantities. This virus, however, is bound and is liberated only if the tissues are subjected to special treatment. Brief reference has already been made to the extraction of tomato bushy stunt virus by the fine milling of infected tomato leaves (Bawden and Pirie, 1943*a*). The present paper describes some of the conditions affecting the liberation of this normally insoluble virus by milling and other treatments.

The broad principles are fairly clear, but it is difficult to be definite about details for different lots of plants behave very differently. The leaf residues contain approximately as much virus as is extracted in the sap, and it is most completely liberated by incubating the residues with a commercial trypsin preparation and then grinding finely. Either incubation with commercial trypsin or grinding will liberate some of the virus, but the relative proportions liberated by the two treatments vary from one lot of plants to another. The component of commercial trypsin preparations responsible for the liberation of virus is soluble and does not diffuse through cellophane, but we have no evidence that it is actually trypsin. For convenience this phenomenon will be referred to as liberation by "trypsin."

We have made no attempt to standardize the cultural and environmental conditions of the host plants, and the variations in results with different batches of leaves presumably have their origin in differences in these conditions. Tomato plants growing at different times of the year vary in their susceptibility to infection with bushy stunt virus, in the type and severity of symptoms they show and in their virus content; they also, of course, vary in their vigour, colour and habit of growth.

We have found no differences between purified virus obtained from the sap and from the leaf residues, but extracts from infected residues have serological properties different from any previously described. Some contain virus in the form of a non-precipitating antigen, while others contain materials that prevent the normal precipitation of the virus by its antiserum.

MATERIALS AND METHODS.

Preparations of leaf residues.

All the work described has been done with young tomato plants of the variety Kondine Red, which were grown in glasshouses with heat but no extra illumination. In the summer months seedlings rapidly pass through their most susceptible period, and to get good systemic infection, with a high virus content, they must be inoculated when not more than 6 in. high. In the winter, plants retain their susceptibility for longer, and the symptoms are usually more necrotic and less chlorotic than in the summer. Leaves are picked from 8–14 days after infection, when the symptoms are well developed. The main stem and bases of the petioles are not included, as these contain only about one-eighth of the virus present in the laminae and their presence greatly complicates all later operations.

The leaves are minced with a domestic meat mincer and the sap expressed by hand through madapollam ; from 100 g. of fresh leaves there is, on an average, 80 g. of sap and 20 g. of residue. If the leaves or the mince have been frozen, or if the residue is minced again after one squeezing, there is a slightly greater yield of sap. Provided the pH is unaltered, the amount of sap that can be expressed by hand is not significantly altered by further or finer grinding. This suggests that most of the cells are sufficiently damaged by freezing and mincing, or by two successive mincings, to liberate their sap, and that the effect of fine milling is not simply to damage further cells.

Sap remaining in the leaf residues can be removed by suspending them in a volume of water equal to that of the sap and squeezing through madapollam. Even with the most infective sap, four such washes have been sufficient to reduce the virus content of extracts below that necessary to give a visible precipitate with antiserum. For the first two washes the water is expressed after a few minutes in contact with the residues, and for the last two it is left in contact for some hours. Material prepared in this way, pressed as dry as possible by hand, will be referred to as washed fibre ; it forms the starting material for the experiments described below.

Milling the washed fibre.

For fine grinding we have used a triple-roller ointment mill (made by the Pascall Engineering Company, London) that grinds 1–2 g. wet weight of fibre per minute. This has three porcelain rollers, cylinders 10 cm. long and 5 cm. diameter, driven by a gear train at 160, 350 and 520 r.p.m. respectively. The middle roller revolves in the opposite direction to the other two, so that material fed between the slow and the middle roller is carried on the undersurface of the middle roller and ground again between this and the fast roller ; it is finally removed by a scraper. The fineness of grinding is controlled by the force holding the rollers together ; this is normally adjusted so that the $\frac{1}{4}$ h.p. motor driving the mill begins to " labour " when a charge of proper consistency is passing through.

Grinding is only effective over a narrow range of water content ; if the charge is too wet it does not cling to the rollers, and if it is too dry there is insufficient lubrication where t' rollers rub together. The moisture content of washed fibre in the usual pH range ·7 to 7·2) is about 70 per cent. This is too low for satisfactory grinding, and one part of water should be added to three of fibre to get the best consistency.

Separation of fluid from milled fibre.

Fluid can be separated from the finely ground fibre by squeezing the paste in a madapollam bag, but by assiduous kneading much of the solid also passes through the bag. This leads to great variations in results, for the cloth becomes clogged and

less porous as the separation proceeds. This phenomenon becomes more pronounced with increasing pH, which increases the water-holding capacity of the ground fibre. Above pH 8 separation of fluid and solid is no longer practicable by squeezing through a cloth. It can be done by centrifugation, but within the pH range used the water content of sediments is so high that this is an unsatisfactory method of separation, for, unless such large volumes of extracting fluid are used that there is excessive dilution of virus, a large proportion of the virus will be in the free fluid remaining in the sediment. Therefore, for quantities of ground fibre between 2 and 15 g., we have used the following method, which gives reasonably reproducible results. A piece of cloth is fastened over the mouth of a 50 or 250 ml. centrifuge tube with a rubber band, and adjusted so that there is enough slack to form a bag in the mouth of the tube. With properly adjusted tension on the band, the system can be centrifuged at 1000 r.p.m. All the fluid that can be separated passes through the cloth in 10 minutes at this speed. The area of cloth over which the material is distributed is always small, so that the recovery of both extract and residue is more nearly quantitative than when separation is done by hand.

Neutralization of fibre.

For testing the effect of enzymes in liberating virus from leaf residue it is necessary to control the pH of the suspension. This needs careful checking, as the pH value of neutralized fibre drifts considerably. The pH of washed fibre is between 5·8 and 6, and very little alkali is needed to take it to 8. However, the pH soon drifts back, and frequent addition of small amounts of alkali is needed during 24 hours at room temperature to stabilize the pH at 7·6 to 8. A total of 3 ml. of N/5 NaOH is needed for 10 g. of washed fibre. Bacteria grow readily on the neutralized suspension, and to prevent this we have used chloroform as an antiseptic. During the neutralization 1 ml. of chloroform is added for each 10 g. of washed fibre ; for ease of stirring during the addition of NaOH, the fibre is suspended in a volume of water equal to twice that of the sap previously expressed from it, i.e. 60–80 ml. per 10 g. Washed fibre neutralized in this way was used in all the incubation experiments described, and all subsequent operations were made with fluids saturated with chloroform.

Methods of testing.

The virus content of the extracts from infected plants has been estimated by titration against antiserum prepared by injecting rabbits with purified bushy stunt virus. Occasional infectivity tests showed that this antigen was active virus ; these were done by the local lesion method, using *Nicotiana glutinosa* as a host plant. In the precipitin tests 1 ml. of antiserum at a constant dilution was added to a series of tubes each containing 1 ml. of extract at varying dilutions. The tubes were then placed in a water-bath at 50° C., with their fluid column half immersed to ensure mixing by convection, and the greatest dilution at which the extract gave a visible precipitate was taken as the end-point. Usually the extracts were diluted serially with a factor of two, and no attempt was made to detect smaller differences.

The sap of plants clarified by centrifugation at 3000 r.p.m. may give unspecific precipitates under the conditions of test, but this is easily recognized by controls with saline or normal rabbit serum. This tendency is reduced if the leaves or sap have been frozen and thawed before centrifuging, or if a little Na_2HPO_4 has been added or the sap has been left for a few hours at room temperature. After such treatments, clarified sap is clear brown and suitable for immediate use in precipitin tests.

Extracts made from milled fibre or from fibre incubated with " trypsin " need further treatments before precipitin tests are a reliable index of their virus content.

The extracts made from milled fibre are deep green, and the chromoprotein is not removed by centrifugation at 3000 r.p.m. even after they have been stored for some days at room temperature. The yield of chromoprotein is higher in the summer than in the winter and its extraction is favoured by low salt content, high pH and fine grinding. These extracts sometimes give bulky unspecific precipitates in the water bath, but they are more often stable, and the chief complication in serological tests is the failure to obtain a specific precipitate with antiserum when the virus content is adequate. Hence the chromoprotein must be removed before precipitin tests are made. This is most conveniently done by freezing for 15 hours at —7° C., which coagulates the chromoprotein but, at the virus concentration, salt content and pH usual in these extracts, does not inactivate the virus (Bawden and Pirie, 1943*b*).

The conditions used for enzymic digestion usually give extracts that are too dilute for satisfactory serological tests ; after incubation with " trypsin " such extracts may also contain material that prevents the precipitation of virus by antiserum. Before testing, the extracts are concentrated and the inhibiting material removed. The fibre and fluid are first separated by centrifuging through cloth as already described. After removing the cloth from the tube, any insoluble material in the fluid is packed by centrifuging for 15 minutes at 3000 r.p.m., when the supernatant fluid is poured off and half saturated with ammonium sulphate. If digestion has been prolonged there is immediate precipitation, but if it has been brief or with little enzyme, precipitation is delayed. The mixture is therefore left for 3 hours at 25° C. to ensure complete precipitation. After centrifuging for 30 minutes at 3000 r.p.m. the pellet is suspended in 1·5 ml. of water for each gramme of fibre extracted, and 1 drop of 10 per cent. acetic acid is added to the thoroughly mixed suspension to bring the pH to 4–4·5. The insoluble material, which contains the inhibitor, is removed by a few minutes' centrifuging at 3000 r.p.m. After neutralization, the supernatant fluid is suitable for testing serologically.

RESULTS.

Liberation of virus by milling and enzymic digestion.

After freezing and centrifuging, extracts made by suspending milled fibre in three times its weight of water are clear and only pale straw colour. Their virus content varies with different lots of plants, as does the virus content of sap. The precipitin titre of such extracts may be the same as that of sap, but more usually it is one-half or one-quarter. Variations in pH between 5 and 7·5 or in the nature and concentration of the buffer solution have had no significant effect on the liberation of virus. A second extraction of the milled fibre yields additional virus, but there is usually a greater liberation if the milling is repeated before the fibre is extracted. With some batches of leaves the amount of virus released by successive millings falls slowly, whereas with others nearly all the virus is released by one milling. Table I illustrates these variations ; in general, more of the virus is liberated by one milling from plants grown in the winter than from those grown in the summer. In making these extracts the milled fibre was soaked in the fluid for 30 minutes before separation ; longer soaking does not bring out significantly more virus.

The amount of virus liberated by incubation with commercial trypsin preparations (British Drug Houses) depends on the pH, amount of " trypsin " and duration and temperature of incubation. We have standardized the last three factors by allowing 0·1 per cent. solutions of " trypsin " to act for 15 hours at 42° C. More intense digestion does not give sufficient extra virus to be detected by the methods of testing used. Column 1 of Table II gives the results of incubating washed fibre at different pH values with " trypsin," and shows a clear optimum between 6 and 7. No attempt has been

TABLE I.—*Virus Content of Sap and Successive Mill Extracts.*

Month of harvesting plants.	Serological titres.			
	Sap.	First mill extract.	Second mill extract.	Third mill extract.
January	256	256	8	0
February	256	180	8	0
March	660	350	60	10
May	64	32	16	8
July	450	64	24	6
September	96	40	20	10
December	80	16	1	0

Washed fibre was milled and extracted twice with 2–3 times its weight of M/50 pH 7 phosphate buffer; the two extracts were mixed and prepared for testing as described in the text. The fibre residue was then milled again and extracted in the same way. The titres given are the observed precipitation end-points of sap or mill extract multiplied by a factor to bring them to the standard ratio of 1 ml. of fluid from 1 g. wet weight of washed fibre.

TABLE II.—*The Effect of pH on the Liberation of Virus from Fibre by Incubation with "Trypsin."*

pH.	Serological titres.		
	1. Extract from fibre incubated with "trypsin."	2. Residues from 1 re-extracted at pH 7.	3. Residues from 2 milled and extracted.
4	2	2	32
5	16	4	32
6	64	0	32
7	48	0	32
8	16	0	24
9	0	0	16
Control fibre milled without previous treatment			8

Titres are reduced to a standard basis of 1 ml. of extract from 1 g. of washed fibre.

made to determine the optimum more closely; the pH is a matter of uncertainty when over 6·5, as there is a considerable liberation of acid during incubation. 1 ml. of buffer solution, made by adjusting a 4 per cent. solution of Na_2HPO_4 to the required pH with acetic acid, is added for each gramme of fibre, but this is insufficient to hold the pH at the required value, and the pH must be adjusted three or four times during the first five hours if it is to be kept constant to within 0·5 of a unit.

Provided adequate amounts of antiseptic have been present throughout the operations on the washed fibre, incubation without the addition of "trypsin" usually fails to liberate virus, although occasionally it has given an eighth as much as has incubation with "trypsin." Incubation with pepsin or papain, with or without cyanide, has liberated no more virus than incubation alone. These two enzymes are not ineffective because they destroy the virus, for provided that the incubation has not been carried out at a pH below 3, virus can still be liberated by subsequent milling or by incubation with "trypsin."

When no more virus can be released by further milling, some can still be obtained by incubation with "trypsin." The amount varies with different lots of leaves and is never large; it seems to be greater with plants grown in the winter. By contrast, from washed fibre that has been incubated with "trypsin" there is a copious liberation of further virus on milling, although no more is liberated by increased incubation. Indeed, extracts with the highest precipitin titres are often obtained by this method,

in spite of the fact that virus has previously been extracted as a result of the incubation with " trypsin." Table III shows that there is greater liberation of virus when incubation with " trypsin " precedes milling than when the sequence is reversed ; it also shows how the discrepancy between the yield of virus by the two methods increases the more often the fibre has been milled before it is incubated with " trypsin."

Even at pH values at which incubation with " trypsin " gives little direct liberation, there is some loosening of the virus from its connection with the fibre. This is shown in Table II. Fibre that has been incubated with " trypsin " on the acid side of the optimum releases some virus when subsequently extracted at pH 7 (Table II, column

TABLE III.—*The Effect of the Sequence of Treatments on the Serological Titres of Successive Extracts.*

Trypsin	64	Trypsin	64	Mill	16	Mill	16	Mill	16	Mill	16
Trypsin	16	Mill	64	Mill	1	Mill	1	Mill	1	Trypsin	64
Trypsin	2	Trypsin	16	Mill	0	Mill	0	Trypsin	32	Mill	4
Trypsin	0			Mill	0	Trypsin	16	Mill	2	Trypsin	0
Mill	128			Trypsin	4	Mill	2	Trypsin	0		
						Trypsin	0				

Each column deals with a separate 2 g. sample from the same lot of washed fibre, which was subjected successively to the treatments listed. After each treatment the fibre was extracted twice with a total of 12 ml. of water ; the two extracts were combined and precipitated with acid and ammonium sulphate before testing serologically ; the residue was then subjected to the next treatment. Titres are reduced to a standard basis of 1 ml. of extract per g. of washed fibre.

2). Column 3 gives the virus contents of extracts made by milling, and here also the effect is clearly shown, for extracts from fibres incubated with " trypsin " at all pH values between 4 and 9 contain more virus than the mill extract of unincubated washed fibre. Incubation without the addition of " trypsin " has never led to a similar increase in the amount of virus liberated by milling, provided that antiseptic precautions were taken ; occasionally incubation alone has reduced the amount of virus liberated by milling.

Properties of virus from sap and residues.

Purified preparations of bushy stunt virus have been made by the method recently described (Bawden and Pirie, 1943*a*) from infective sap, and from the extracts prepared by subjecting the residues to fine grinding or incubation with " trypsin." Preparations from the two sources have not been found to differ in any significant manner.

TABLE IV.—*Activity of Purified Virus Preparations made from Sap and Fibre.*

Source of virus.	Serological titre.	Infectivity. Average number of lesions per leaf at—		
		10^{-5}.	10^{-6}.	10^{-7}.
Sap	1/300,000	66	12	1
Milled fibre	1/300,000	60	15	2
Sap	1/300,000	90	17	2
Milled fibre	1/300,000	78	15	1
Sap	1/300,000	115	36	–
Milled fibre	1/300,000	103	38	–
Trypsined fibre	1/300,000	99	23	–
Sap	1/400,000	41	19	4
Milled fibre	1/400,000	49	26	6

They have similar serological activity and infectivity (Table IV), and similar phosphorus and carbohydrate contents; they crystallize in the same form and behave in the same way during crystallization.

Measurements of sedimentation constants have not been made, but comparisons of the weight of virus compacted into a pellet when preparations from sap and mill extracts are ultracentrifuged in the same conditions show that there is no gross difference. In making such a comparison the pH of the virus solutions needs careful control. McFarlane and Kekwick (1938) found that variations in pH between 2 and 9 had no effect on the rate at which the boundary moves during centrifugation. It does, however, greatly affect the rate at which the concentrated virus at the bottom of the tube compacts into a coherent pellet. In our tests 0·15 per cent. virus solutions in M/40 phosphate or acetate buffer were centrifuged in the 7·5 ml. tubes of an air-driven centrifuge of the type described by Masket (1941). The fluids were centrifuged for 30 minutes at 40,000 r.p.m. (90,000 R.C.F.), followed by 30 minutes during which the rotor was allowed to coast and the speed fell to 30,000 r.p.m. This degree of centrifugation at the isoelectric point (pH 4·2) packs all the virus into a compact pellet, which remains in the tube when the fluid is decanted. By contrast, at pH 8 there is only a trace of a compacted pellet, and almost all the virus lies at the bottom of the tube as a dense, mobile fluid; more prolonged centrifugation at this pH compacts more of the virus. With increasing pH from 4 to 8, the ratio of pellet to dense fluid falls steadily. Below pH 4·2 the boundary between supernatant fluid and sediment is less definite, and the sediment, although easily visible, is so limp that it is apt to slide out of the tube when the fluid is decanted.

Comparisons have been made at pH 6 of the ratio of compacted to uncompacted virus in purified virus preparations made from sap and mill extracts of fibre. No differences were found, and the activities of the compacted and uncompacted virus did not differ.

Virus as a non-precipitating antigen.

Mill extracts of washed fibre from different batches of infected plants vary in their behaviour in precipitin tests, but they usually give no specific precipitate with virus antiserum until the chromoprotein has been removed. This can be removed satisfactorily in any of five ways: by incubation with "trypsin"; by centrifuging at 12,000 r.p.m.; or by low speed centrifugation after the extracts have been frozen, heated to 60° C. or acidified to pH 4. The untreated extracts fail to precipitate with virus antiserum because of the presence of virus combined with chromoprotein to form a non-precipitating complex, which also interferes with the precipitation of free virus. This complex has serological properties resembling those of the non-precipitating antigens produced by Bawden and Kleczkowski (1941, 1942) by heating bushy stunt virus with serum albumin.

Table V illustrates how the removal of chromoprotein alters the reactions of a deep green mill extract with virus antiserum. The chromoprotein separated by freezing or by centrifugation at 10,000 r.p.m., often resuspends in water to give a stable suspension. If it is resuspended in its own supernatant fluid, the resulting mixture resembles the original mill extract and is not precipitated by virus antiserum. As chromoprotein from infected leaves prevents precipitation of free virus, there is no reason to suppose that all, or even most, of the virus in the mill extract was originally in the form of a complex that was split by freezing or high-speed centrifugation. The inhibition of precipitation is specific, and if the chromoprotein is added to solutions of antigens other than bushy stunt virus, e.g. tobacco necrosis viruses or serum

TABLE V.—*The Effect of Freezing on the Serological Behaviour of Extracts from Milled Fibre.*

Time after mixing with serum.	Mill extract. Dilution. 1/1.	1/2.	1/4.	1/8.	1/16.	Extract after removal of chromoprotein by freezing. Dilution. 1/1.	1/2.	1/4.	1/8.	1/16.
30 min.	—	—	—	—	—	++	+	—	—	—
2 hours	—	—	—	—	—	++++	+++	++	+	—
8 ,,	—	—	—	—	—	++++	++++	+++	++	+

Plus signs indicate the degree of precipitation; minus signs, no precipitation. 1 ml. of extract at each dilution mixed with 1 ml. of virus antiserum at 1/50.

TABLE VI.—*Inhibition of the Serological Precipitation of Free Virus by Virus-chromoprotein Complex.*

Time after mixing with serum.	Amount of purified virus added to mill extract (in μg.). 200.	100.	50.	25.	12.	6.	Amount of purified virus added to saline (in μg.). 200.	100.	50.	25.	12.	6.
10 min.	—	—	—	—	—	—	++	+	—	—	—	—
20 ,,	++	—	—	—	—	—	+++	++	+	—	—	—
1 hour	+++	+	—	—	—	—	++++	+++	+++	++	+	—
4 hours	++++	+++	++	—	—	—	++++	++++	++++	+++	++	+

Description in text.

TABLE VII.—*Inhibition of Serological Precipitation in Extracts from Fibre Incubated with "Trypsin."*

Time after mixing with serum.	Trypsin extract. Dilution. 1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	Extract after centrifugation at pH 4. Dilution. 1/2.	1/4.	1/8.	1/16.	1/32.	1/64.
1 hour	—	—	—	—	—	—	++++	+++	++	+	—	—
2 hours	—	—	—	—	+	—	++++	++++	+++	++	+	—
6 ,,	—	—	—	++	++	+	++++	++++	++++	+++	++	+

1 ml. of extract at each dilution mixed with 1 ml. of virus antiserum at 1/50.

globulin, these precipitate normally when the homologous serum is added. Also, chromoprotein from healthy tomato leaves does not inhibit the precipitation of bushy stunt virus by its antiserum. Hence the phenomenon is not simply a result of chromoprotein interfering in some mechanical and unspecific way with the separation of floccules.

If the chromoproteins that separate when mill extracts are frozen, acidified or centrifuged at 12,000 r.p.m. are suspended in water, no more virus is extracted than would be contained in their free water. After thorough washing, however, the protein from a non-precipitating extract still contains bound virus. Incubation with " trypsin " liberates this virus in a form that is precipitated by virus antiserum; the amount liberated varies with different lots of chromoprotein, but 0·1 mg. from 10 mg. of chromoprotein is an average value. The virus content of chromoprotein derived from later mill extracts is always less than that of chromoprotein from the first extracts.

The washed precipitates of chromoprotein from infected plants combine specifically with virus antibodies. This can be shown by mixing suspensions with diluted antiserum, centrifuging for 15 minutes at 12,000 r.p.m. after incubation, and then testing the supernatant fluid for precipitating antibodies; 5 mg. of washed chromoprotein is usually sufficient to absorb all the precipitating antibodies from 0·2 ml. of bushy stunt virus antiserum. Heterologous antisera still precipitate strongly after such absorption, and there is no comparable absorption of bushy stunt virus antibodies by chromoprotein from uninfected fibre. Washed chromoprotein from infected plants is antigenic and produces antibodies that precipitate free bushy stunt virus; a rabbit injected with 15 mg. of a non-precipitating complex of chromoprotein and virus gave a serum with a precipitin titre of 1/80 when tested against purified bushy stunt virus at 0·05 mg. per ml.

We have no evidence that the virus occurs naturally in the fibre as a complex with chromoprotein, for extracts with serological properties similar to those of mill extracts from infected leaves can be made by milling fibre from healthy leaves to which purified bushy stunt virus has been added. For example, 4 g. of washed fibre from healthy tomato leaves was mixed with 1·5 ml. of pH 7 0·3 M phosphate buffer containing 2 mg. of purified virus, passed through the mill twice and extracted with 10 ml. of water; this green extract did not precipitate with virus antiserum, but after freezing it precipitated to give an end-point of 16. An extract from an identical mixture that was passed through the mill four times likewise only precipitated after freezing; it then gave an end-point of 8. By contrast, when 4 g. of the uninfected fibre was milled twice with phosphate buffer and then allowed to stand for an hour mixed with 10 ml. of water containing 2 mg. of virus, the green extract was precipitated by virus antiserum and gave an end-point of 32 both before and after freezing. Thus the non-precipitating complex is not formed merely by mixing virus with milled fibre, but only when the two are passed through the mill together. A comparison of the titres also shows that the recovery of virus from the milled mixtures is incomplete, and that repeated milling reduces the amount recovered. This is comparable to the effect already illustrated in Table III. Some, but by no means all, of this missing virus can be liberated from the residual fibre and the separated chromoprotein by incubation with " trypsin " at pH 6·5. Incubating infected fibre with " trypsin " has already been shown to increase the amount of virus liberated by milling. Experiments were therefore made with uninfected fibre to see whether a previous incubation with " trypsin " affected the recovery of purified virus added before milling. There was, however, the same loss with washed fibre and with fibre incubated with " trypsin." These effects are not peculiar to the tomato plant; when bushy stunt virus is milled with fibre from healthy leaves of French bean (*Phaseolus vulgaris*, var. Canadian Wonder) there is a

similar loss of virus, and the extracts do not precipitate with virus antiserum until the chromoprotein is removed.

The behaviour of mill extracts from different lots of infected plants depends, not on the concentration of chromoprotein, but on the ratio of free virus to virus combined with chromoprotein as a non-precipitating complex. Extracts made from plants grown in the winter often contain little chromoprotein but much virus, and these precipitate with virus antiserum, though more slowly than after the removal of chromoprotein. With much chromoprotein and little free virus, extracts not only fail to precipitate with virus antiserum, but additional free virus can be added without causing precipitation. This is shown in Table VI. A deep green non-precipitating extract was used, which after removing the chromoprotein complex had a precipitation end-point of 16, indicating a free virus content of about 0·05 mg. per ml. 1 ml. samples of the extract were mixed with 1 ml. of virus antiserum at 1/50 and, after incubation for 1 hour at 50° C., varying amounts of purified virus were added to the mixtures. A control series of tubes was also used in which virus was added to mixtures of saline and antiserum. It will be seen that the presence of chromoprotein did not prevent precipitation, but that the concentration of free virus had to be doubled before precipitation occurred, and then it was slower and smaller than in the control tubes containing equivalent amounts of virus.

The results of Table VI show that after mixtures of chromoprotein and antiserum have been incubated, precipitation will occur if the content of free virus is increased sufficiently. This suggests that although the virus-chromoprotein complex combines with antibody, it is not preventing precipitation by leaving no antibody available for combination with free virus. Also, precipitation does not occur if the antiserum is increased to many times the amount that can be absorbed by the virus-chromoprotein complex present. Thus the inhibition of precipitation cannot be attributed to failure of the free virus to combine with antibody, but rather to some factor affecting the normal processes that intervene between combination of antigen and antibody and the separation of visible floccules. The virus-chromoprotein complex is readily sedimented by centrifugation at 12,000 r.p.m., so that the particles to which the virus is bound are much larger than the virus itself, although they are still too small to settle under gravity. Probably only a small part of the surface of these large particles is involved in combination with virus, and so most of it will remain unaffected when the virus combines with antibodies. Most of the hydrophylic groups will remain uncovered, and its failure to precipitate with virus antiserum is not unexpected. Presumably these complexes prevent the precipitation of free virus by antiserum either by preventing the formation of a regular lattice structure of antigen-antibody particles, which is believed to be the first phase of precipitation, or else they diminish the tendency of these aggregates to combine with one another so that they do not aggregate further to give the second stage that leads to the separation of visible floccules.

Only a small part of the total virus need be in the form of a non-precipitating antigen to inhibit precipitation completely. From mill extracts that failed to precipitate with virus antiserum, more than 7/8 of the virus has been found in the supernatant after freezing, and less than 1/8 has been recovered from the chromoprotein by incubation with " trypsin."

Non-specific inhibition of precipitation.

Extracts made from infected fibres that have been incubated with " trypsin " usually behave anomalously when used in precipitin tests with virus antiserum. Although they contain so much virus that they would give a visible precipitate in normal conditions, they may either fail to precipitate, or they precipitate only after

long incubation with antiserum, and then only at high dilutions. If the extracts are brought to pH 4 and centrifuged, the supernatant fluids after neutralization precipitate normally with antiserum. This phenomenon is illustrated in Table VII.

This type of inhibition does not depend on the presence of virus in a non-precipitating form, for extracts with similar inhibitory effect can be made from healthy tomato leaves or from leaves of *Phaseolus vulgaris* and tobacco. Also, the extracts can inhibit the precipitation of more than one antigen-antibody system. The inhibition is therefore not type-specific, but is caused by the presence of material that acts as a " protective colloid " and stabilizes the system. With mill extracts inhibition depends on the ratio of non-precipitating virus complex to free virus, and if an extract fails to precipitate when concentrated it will not precipitate after dilution. Table VII shows that the non-specific inhibition depends more on the actual concentration of the inhibitor, for equal dilution of virus and inhibitor leads to precipitation. There is, however, a critical amount of virus that can be stabilised by a given concentration of inhibitor, and a virus-antibody system that is giving no precipitate will precipitate if the virus content is increased.

The amount of inhibitor that can be extracted from washed fibre depends on the treatments given. Very little is obtained unless the fibre is incubated with " trypsin," and the amount is further increased if it is milled before incubating with " trypsin." This is shown in Table VIII. The precipitate that separates at pH 4 from inhibiting

TABLE VIII.—*The Effect of Different Treatments in Producing Inhibitor from Fibre.*

Treatment.	Inhibition titre.
Milled	1
Trypsined	4
Milled, then trypsined	8
Milled, trypsined and milled again	8
Trypsined, then milled	4

The inhibition titre is the highest dilution at which the solution completely prevented precipitation of added virus. After each treatment the fibre was extracted and precipitated at pH 4 as described in the text. The acid precipitates from the successive treatments on each sample of fibre were mixed, washed, dissolved, and reprecipated before testing.

extracts made from infected fibre contains some virus. Part of this can be removed by washing with water at pH 4, but the removal is more complete if the resuspended precipitate is brought to pH 7 and again precipitated with acid. The materials used for the comparison given in Table VIII were all prepared in this way, and the second precipitate at pH 4 was taken up in pH 7 M/100 phosphate buffer, using 1·5 ml. for each gramme of washed fibre, and centrifuged at 3000 r.p.m. before testing serologically. 1 ml. of the extracts at different dilutions were mixed with 1 ml. of bushy stunt virus antiserum at 1/100, and incubated for 1 hour at 50° C. before adding 0·05 mg. of bushy stunt virus.

We have made some preliminary attempts to purify this inhibitor. After several precipitations with ammonium sulphate and acid, 0·2 mg. of the best preparations have inhibited the precipitation of 0·05 mg. of bushy stunt virus with antiserum. These preparations contain both protein and carbohydrate.

DISCUSSION.

The phenomena described in this paper are not peculiar to tomato plants suffering from bushy stunt. Washed fibre from tomato and tobacco plants infected with tobacco mosaic virus, from tobacco plants infected with tobacco ringspot virus, and

from tobacco and bean plants infected with tobacco necrosis viruses, contains virus that is set free by fine milling or by incubation with " trypsin." Mill extracts from all such infected plants contain some virus combined with chromoprotein to give complexes that do not precipitate with homologous virus antiserum, and extracts from plants suffering from tobacco ringspot or tobacco necrosis usually fail to precipitate with virus-antiserum unless the chromoprotein is removed.

Virus is liberated from washed fibre by other means of grinding than the one described in this paper. A large mill with three rollers 29 cm. long and 15 cm. in diameter and running at 18, 45 and 180 r.p.m. respectively behaved similarly to the smaller mill. Some virus was also liberated from washed fibre by grinding for 10 minutes in an end-runner mill and in a " Waring Blendor," but not by passage through a Latapie mincer. Freezing leaves and passing them through a meat mincer damages most of the cells sufficiently to allow the sap to escape. The further effects of fine grinding probably result from the mechanical destruction of cell components that have withstood mincing, so that virus previously held by them is allowed to diffuse away in solution. During milling there is a continuous release of starch and chromoprotein, which suggests that one of the main components being affected by the milling is the plastids.

Incubation with " trypsin " increases the amount of virus set free by milling, and releases further virus after milling has become ineffective. This suggests that virus is held in the fibre in two different ways, and that some is attached to materials, or contained within structures, which are disintegrated by milling but are not affected by " trypsin," and that other virus is attached to materials which are destroyed by " trypsin " but not by milling. The second type of bound virus would resemble that found in the mill extracts in combination with chromoprotein. We have no grounds for suggesting that virus in the fibre occurs combined with chromoprotein, for combination between the two can be brought about by milling fibre to which free virus has been added. However, this fact does not exclude the possibility that there is some combination between the two in the infected plant.

It is clear that milling has at least two actions on the virus. First, virus is liberated from structures or materials from which it did not previously diffuse at all readily. Secondly, virus is being attached to chromoprotein. It is possible that virus is also being attached to other materials, for as the extent of the milling is increased the amount of virus that can be released by subsequent incubation with " trypsin " falls. This may be due to actual destruction of the virus, perhaps by local, intense overheating between the rollers. There is no appreciable rise in the general temperature of the fibre during grinding, but it is well known from work on the rubbing together of unlubricated, or slightly lubricated, surfaces that very high local temperatures exist transiently (Bowden and Ridler, 1936). Complexes with serological behaviour similar to those of the virus-chromoprotein complex can be made by heating bushy stunt virus with serum albumin (Bawden and Kleczkowski, 1941), and this could be advanced as an argument in favour of the observed effects being caused by heating. On the other hand, bushy stunt virus is very sensitive to heat (Bawden and Pirie, 1943*a*), and the virus isolated after milling is as infective as virus isolated from the sap. Neither argument, however, is conclusive, for in the earlier tests the virus was exposed to temperatures of below 100° C. for some minutes, whereas during milling it is reasonable to postulate temperatures much higher but lasting for very short periods, and nothing is known about the behaviour of proteins in such conditions.

Although destruction of virus during milling cannot be excluded, it seems more likely that the loss is due to the attachment of virus to materials not destroyed by milling or by " trypsin." If this is so, it also suggests that virus may be bound to the

fibre by linkages that are not affected by any treatments yet used, and that other methods of extraction may liberate still more virus from infected plants. Other fractions of the minced leaves may also be sources of virus. The deep green precipitate, consisting largely of fragments of chloroplasts, which separates when sap is centrifuged at low speed, is one obvious possibility. Milling has never liberated any virus from this material, but incubation with " trypsin " has occasionally liberated considerable quantities.

Information about the different ways that virus occurs in infected plants is of interest because of its bearing on the site of virus multiplication. Previous work had suggested that virus was limited to the sap, and attempts to culture plant viruses have usually been made with media containing sap. The fact that the solid residues contain virus raises the possibility that synthesis of virus occurs in the insoluble fraction of cells, from which it passes into the sap, and that any " factors " essential for virus multiplication should be sought for in this fraction rather than in the sap. Bushy stunt virus from sap seems to be identical with that from the solid residues, and the experiments described in this paper do not indicate which is the more likely to be the first formed. However, experiments with tobacco mosaic virus, which will be described later, suggest that the virus obtained from the fibre is a precursor of that found in the sap.

SUMMARY.

After the sap has been expressed from minced tomato leaves infected with tomato bushy stunt virus, the solid residues contain approximately as much virus as the sap. This virus is most effectively liberated by incubating the residues with a commercial trypsin preparation and then passing them through a roller mill ; some virus is liberated by either treatment alone. Incubation with " trypsin " greatly increases the amount of virus liberated by milling, whereas extended milling reduces the amount liberated by " trypsin."

Purified preparations of virus from sap and from the solid residues have similar properties. Extracts of milled fibre contain some virus combined with chromoprotein to form a non-precipitating antigen ; such extracts do not precipitate with virus antiserum until the chromoprotein has been removed. Non-precipitating complexes of virus and chromoprotein can be formed by milling fibre of uninfected plants to which purified virus is added.

Extracts of fibre from healthy and infected leaves, which has been incubated with " trypsin," contain material that inhibits the precipitation of bushy stunt virus by its antiserum.

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REFERENCES.

Bawden, F. C., and Kleczkowski, A.—(1941) *Brit. J. exp. Path.*, **22**, 208.—(1942) *Ibid.*, **23**, 169.

Bawden, F. C., and Pirie, N. W.—(1943*a*) *Biochem. J.*, **37**, 66.—(1943*b*) *Ibid.*, **37**, 70.

Bowden, F. P., and Ridler, K. E. W.—(1936) *Proc. Roy. Soc. A.*, **154**, 640.

McFarlane, A. S., and Kekwick, R. A.—(1938) *Biochem. J.*, **32**, 1607.

Masket, A. V.—(1941) *Rev. Sci. Inst.*, **12**, 277.